



Studies on mating in the budding yeast

Citation

Huberman, Lori Bromer. 2013. Studies on mating in the budding yeast. Doctoral dissertation, Harvard University.

Permanent link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:11181149>

Terms of Use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#).

[Accessibility](#)

Studies on mating in the budding yeast

A dissertation presented

by

Lori Bromer Huberman

to

The Department of Molecular and Cellular Biology

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biology

Harvard University

Cambridge, Massachusetts

June 2013

© 2013 *Lori Bromer Huberman*

All rights reserved

Studies on mating in the budding yeast

ABSTRACT

Budding yeast are capable of existing in both a haploid and diploid state. Haploid cells have two mating types, *MATa* and *MAT α* . When cells from the two mating types come in contact they signal using reciprocal pheromones and pheromone receptors, starting a regulated pheromone response that includes transcription of pheromone-response genes, polarization in the direction of highest pheromone concentration, and cell cycle arrest. Once cells have chosen a mating partner, they must fuse their cell walls, plasma membranes, and nuclei to form a single diploid cell.

We studied two aspects of this process. The first is the role of differentiation in gene expression between *MATa* and *MAT α* cells in controlling mating type and intercellular communication. We created cells that regulate gene expression like one mating type, but express the pheromone, pheromone receptors, and related genes of the opposite mating type. Through characterization of these cells, we identified a *MAT α* -specific gene that blocks the pheromone produced by *MATa* cells, which we call *AFB1* for a-factor barrier. We also show that while changing the regulation of various genes involved with pheromone induction does not cause sterility, it can greatly reduce mating efficiency, providing insight into the robustness of the pheromone response.

Second, we studied the regulation of cell wall fusion during mating. The cell wall provides an elastic force to resist the turgor pressure of the cell, preventing lysis. During cell

wall fusion, the two cells must dissolve their cell walls specifically at the point of contact without exposing their plasma membranes to the extracellular environment, but the mechanism of this regulation is unknown. We provide support for the hypothesis that cell wall fusion is controlled through contact-limited diffusion of cell wall remodeling enzymes. When cells are unattached, secreted cell wall remodeling enzymes diffuse into the extracellular environment, weakening but not breaching the cell wall. However, when two cells are attached, the cell wall remodeling enzymes become trapped between the cell walls, specifically increasing the concentration of these enzymes at the point of contact, and causing cell wall dissolution.

Table of Contents

Title page	<i>i</i>
Copyright page	<i>ii</i>
Abstract	<i>iii</i>
Table of contents	<i>v</i>
List of figures	<i>vi</i>
List of tables	<i>viii</i>
List of supplemental movies	<i>ix</i>
Acknowledgements	<i>x</i>
Chapter 1 <i>Introduction</i>	<i>1</i>
Chapter 2 <i>Genetically engineered transvestites reveal novel mating genes in budding yeast</i>	<i>44</i>
Chapter 3 <i>Polarized secretion and restricted diffusion of cell wall remodeling enzymes induces cell wall dissolution in pheromone-stimulated yeast cells</i>	<i>102</i>
Chapter 4 <i>Discussion, Conclusions, and Future Directions</i>	<i>142</i>
Appendix <i>Supplemental Movies</i>	<i>156</i>

List of Figures

Figure 1.1.	Life cycle of the budding yeast.	4
Figure 1.2.	Pheromone response pathway.	7
Figure 1.3.	Cell wall fusion.	13
Figure 1.4.	Plasma membrane fusion.	17
Figure 1.5.	Control of haploid mating type by the <i>MAT</i> locus.	23
Figure 1.6.	<i>MATa</i> - and <i>MATα</i> -specific genes.	25
Figure 2.1.	Wild-type mating types and transvestite mating types.	47
Figure 2.2.	Transvestite/wild-type diploids are able to proceed through the cell cycle.	51
Figure 2.3.	Transvestite cells can mate.	52
Figure 2.4.	a -playing- α cells respond to a -factor	54
Figure 2.5.	a -playing- α cells produce too little α -factor	56
Figure 2.6.	Increasing the α -factor production of a -playing- α cells increases mating efficiency.	57
Figure 2.7.	<i>AFB1</i> encodes a novel a -factor barrier protein.	60
Figure 2.8.	α -playing- a cells shmoo in response to α -factor.	62
Figure 2.9.	<i>AFB1</i> is partially responsible for the low mating efficiency of α -playing- a cells.	73
Figure 2.10.	Expressing <i>BAR1</i> under <i>P_{FUS1}</i> * does not decrease the mating efficiency of <i>MATa</i> cells.	76
Figure 2.11.	α -playing- a cells arrest only transiently in response to pheromone stimulation.	77
Figure 2.12.	Manipulating the genes expressed at the <i>MAT</i> locus causes extended arrest in α -playing- a cells in response to pheromone stimulation.	79
Figure 2.13.	The control of pheromone-induced cell cycle arrest in α -playing- a cells is complex.	81
Figure 2.14.	Mating is not robust to changes in pheromone production.	83
Figure 3.1.	Secretion in isotropically growing and polarized cells	107
Figure 3.2.	Model: Pheromone-stimulated cell wall dissolution is the result of a contact-driven increase in the local concentration of cell wall remodeling	109

enzymes

Figure 3.3.	Pheromone-stimulated cell lysis is dependent on attachment to a non-permeable surface	112
Figure 3.4.	Pheromone-stimulated cell lysis in bulk culture occurs mainly in cell clumps.	113
Figure 3.5.	Bar1 produced by wild-type <i>MATa</i> cells reduces the α -factor concentration and cells with essentially infinite space to expand can polarize away from the non-permeable surface.	115
Figure 3.6.	Increasing α -factor concentration increases the tightness of polarization and lysis frequency of <i>MATa bar1Δ</i> cells.	118
Figure 3.7.	Benomyl arrested cells rarely lyse in the flow chamber.	119
Figure 3.8.	1M sorbitol protects cells from pheromone-induced cell lysis.	122
Figure 3.9.	The deletion of the putative glucanases Scw4 and Scw11 mitigates pheromone-induced cell lysis	124
Figure 3.10.	The deletion of <i>FUS1</i> and <i>FUS2</i> mitigates pheromone-induced cell lysis	126

List of Tables

Table 2.1.	Genes identified as differentially expressed using RNA sequencing	63
Table 2.2.	Change in expression of genes in the region identified by Steden <i>et al.</i> 1989 between <i>MATa bar1Δ</i> and <i>P_{BAR1}-BAR1</i> α -playing-a cells.	70
Table 2.3.	Strains used in Chapter 2	95
Table 3.1.	Strains used in Chapter 3	135

List of Supplemental Movies

Movie A1.	<i>MATa</i> cells are capable of enduring arrest in response to pheromone stimulation.	157
Movie A2.	α -playing-a cells arrest only transiently in response to pheromone stimulation.	157
Movie A3.	Pheromone stimulated cells lyse when tightly apposed to a non-permeable surface	157
Movie A4.	1M sorbitol protects cells from pheromone-induced cell lysis.	157

ACKNOWLEDGMENTS

The past 6 years have been both more rewarding and more difficult than I imagined upon entering graduate school. I've learned a tremendous amount and truly grown as both a scientist and a person. This would not have been possible without the guidance and support of an extremely large number of people. First of all, I need to thank my advisor, Andrew Murray. He gave me the freedom to work on a project that excited me and was there to help me along when problems arose. I will always appreciate his ability to think of the experiments that really cut to the chase, and I hope I will be able to take that with me as I move on. Along with Andrew, I've had 5 wonderful committee members over the years who gave invaluable advice: Craig Hunter, Erin O'Shea, Rich Losick, Rachelle Gaudet, and Vlad Denic.

The Murray lab is a great place to work. The lab members are always ready to put down their pipettes to answer a question, and I definitely took advantage of that. Although I got general help from just about everyone that I've overlapped with, there are several people who I need to thank specifically. Thanks to John Koschwanetz, Mary Wahl, and Wolfram Möbius who helped me with demystifying UNIX command line to analyze my RNA sequencing data. I'd also like to thank Quincey Justman who taught me a lot about microscopy over the years. Thanks to Natalie Nannas and Derek Lau: two wonderful friends who made grad school fun for the many years we were in lab together. It's been an honor to be part of the intellectual community at Harvard as part of MCB and FAS Center for Systems Biology. Thanks particularly to the entering class of 2007. I also need to thank the National Science Foundation and the Ashford Fellowship for funding over the years.

As much help and support as I've had at Harvard, I also owe many thanks to all the people outside of lab who helped keep me happy and sane. I'd like to thank Cambridge Sports

Union who provided me with friends, exercise, and food over the years along with the New England Orienteering Club. I'd specifically like to thank Brendan Shields who I've had the privilege of being in school with for the past 12 years; he has been a wonderful friend for all of them. Thanks also to the Metropolitan Wind Symphony and the Dudley House Orchestra for satisfying my flute-playing needs. In particular, I need to thank Laura Ross who I get to sit next to every Wednesday evening and has been a great friend since we met as freshmen at MIT.

This never would have been possible (for all sorts of reasons) without the help and support of my parents, Ruth Bromer and Joseph Huberman, who have been there for me whenever I've needed them for the past 28 years. They always encouraged me to do what makes me happy, have supported me in my every endeavor even as my work became increasingly incomprehensible, and coined the term yeastie beasties. I would also like to thank, Carlie Huberman, for being a great sister. I owe a tremendous thank you to my partner, Stephen Granger-Bevan, who even at the end of our first date walked me into lab at midnight to run a gel. He's been a wonderfully supportive partner, and the journey of grad school would have been a lot longer without him. Last but not least, I have to thank my own personal *AFBI* (a fast boy), Presto, who has been a constant source of love, loyalty, and humor.

CHAPTER 1

Introduction

ABSTRACT

The budding yeast, *Saccharomyces cerevisiae*, is capable of both sexual and asexual reproduction. When two asexually reproducing haploid cells of opposite mating types come in contact they are capable of fusing to form a single diploid. The mating type of a yeast cell is controlled by transcription factors expressed at the *MAT* loci. Cells that express the transcription factors encoded at the *MAT_a* locus express the pheromone **a**-factor and the α -factor receptor Ste2 as well as several other *MAT_a*-specific genes, while cells that express the transcription factors encoded at the *MAT α* locus express the pheromone α -factor and the **a**-factor receptor Ste3 as well as several other *MAT α* -specific genes. When *MAT_a* and *MAT α* cells come in contact with one another, they signal through the reciprocal receptors to induce three, interconnected, pheromone-induced responses. The first is the expression of pheromone response genes. The second is arrest in the G1 phase of the cell cycle. The third response is polarization of the cell in the direction of highest pheromone concentration to form a mating projection known as a shmoo. Once two cells have successfully signaled towards each other, they must go through the process of cell fusion, which has four stages. The first stage is agglutination or attachment of the cell walls of the two cells at their shmoo tips. Next, the two cells fuse their cell walls, which must be carefully regulated both spatially and temporally to ensure that cells do not expose their plasma membranes to the extracellular environment and cause osmotic lysis. Once the two cell walls have fused, the cells fuse their plasma membranes to cause cytoplasmic mixing. Finally, the haploid nuclei of the two cells must travel towards each other and fuse to form a single diploid nucleus. Taken together, mating in the budding yeast provides an excellent model system for biological processes important in higher eukaryotes.

INTRODUCTION

The life cycle of the budding yeast, *Saccharomyces cerevisiae*, makes it an excellent model organism. Budding yeast cells have three cell types controlled by the expression of transcription factors at the *MAT* locus, which can be divided into two classes. The first class is haploid. This class has two cell types, called mating types: *MATa* and *MAT α* . When separate, the haploids of commonly used lab strains can replicate asexually by going through mitosis and generating daughters that are genetically identical to their mothers. However, when cells of opposite mating types come in contact with each other, they can fuse to become a single diploid cell. This diploid can replicate asexually via mitosis to create identical daughters or, if starved for nitrogen and grown on a non-fermentable carbon source, can sporulate via meiosis and generate four haploid cells – two of each mating type (Figure 1.1). The ability of budding yeast to replicate in both a sexual and asexual fashion and their genetic tractability has made it possible to use them to study many aspects of biology¹⁻⁴. However, we are most interested in the mating of the budding yeast: the process by which two haploid cells fuse to become a single diploid.

In order for two haploid cells to mate, they must first determine that a potential partner exists. To do this, yeast signal through reciprocal pheromones and pheromone receptors⁵. The yeast pheromones are small molecules that are secreted from the cell. *MATa* cells secrete the pheromone **a**-factor, a 12-amino acid, farnesylated peptide⁶. *MAT α* cells secrete the pheromone α -factor, which is a 13-amino acid peptide without any added moieties^{7,8}. These pheromones are ligands for reciprocal pheromone receptors^{9,10}. *MATa* cells express the α -factor receptor, Ste2 on their surface^{9,11}, and *MAT α* cells express the **a**-factor receptor, Ste3 on their surface¹⁰. The pheromone receptors are G-protein coupled receptors¹² and, when bound to pheromone, signal through their associated G-proteins to activate a mitogen-activated protein (MAP) kinase

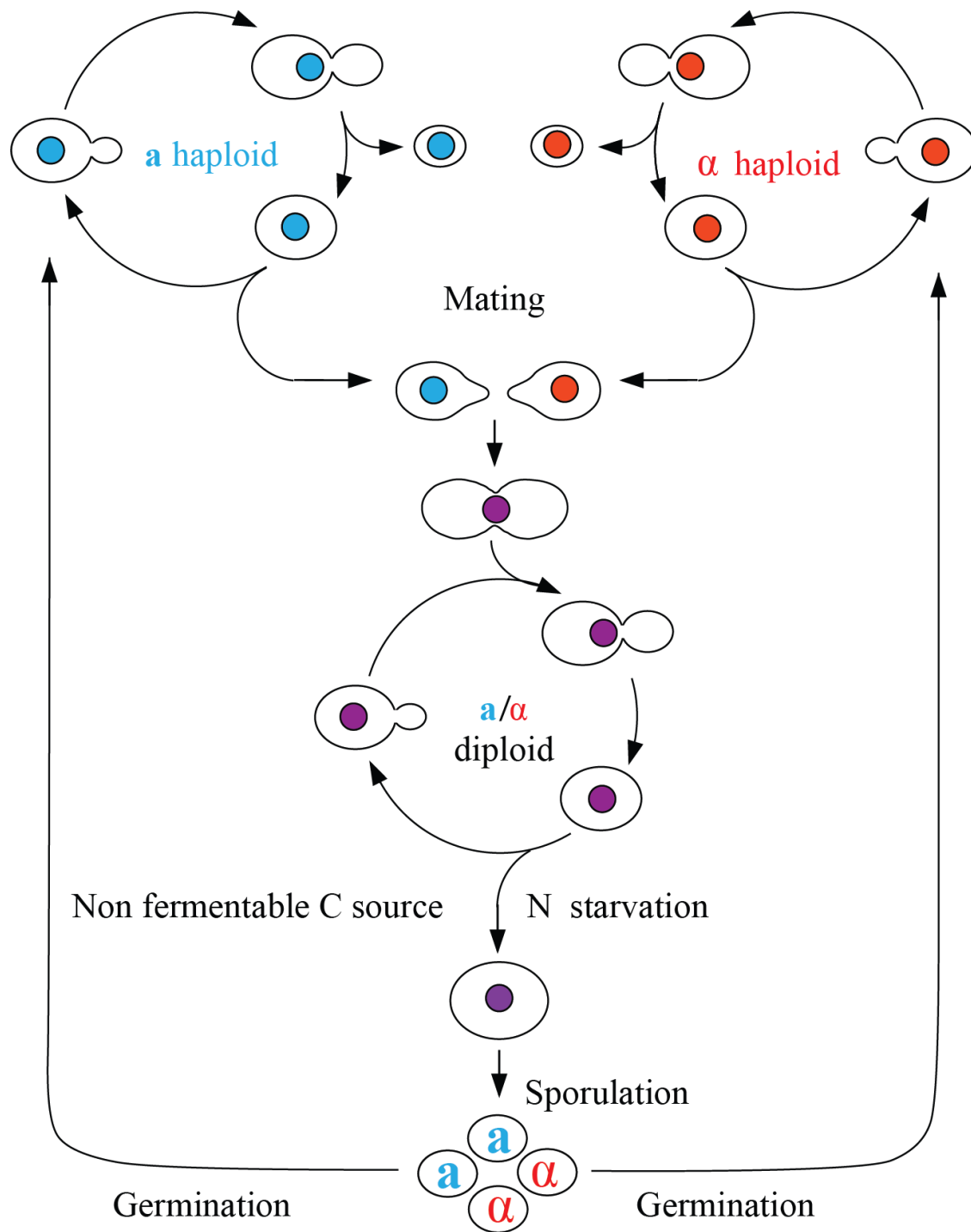


Figure 1.1. Life cycle of the budding yeast. Budding yeast can exist in either a diploid or a haploid state. There are two haploid mating types, which can both replicate asexually by proceeding through mitosis. If they are brought in contact with each other, they signal to each other and fuse to form a single diploid. Diploids can replicate asexually by proceeding through mitosis. However, if they are fed a non-fermentable carbon source and starved for nitrogen, they proceed through meiosis, producing four haploid spores – two of each mating type – and starting the process over again. (Modified from A. Murray with permission.)

cascade⁵, induce cell cycle arrest in G1¹³, and polarize in the direction of highest pheromone concentration to form a mating projection known as a shmoo^{14,15}. Although the pheromones and receptors differ between the mating types, all signaling downstream of the pheromones and receptors is thought to be the same in *MATa* and *MATα* cells⁵.

Once two cells have polarized towards each other, they fuse to form a single diploid cell. To do this, cells must fuse three envelopes: the cell wall, the plasma membrane, and the nuclear envelope¹⁶. The yeast cell wall is a complex network of proteins and carbohydrates that, among other things, provides an elastic force to resist the turgor pressure that would otherwise cause the cell to lyse¹⁷. To fuse the cell walls, both cells dissolve their cell walls at the point of contact while simultaneously fusing the boundaries of the cell walls to form a single, continuous structure. Once a hole has formed between the two cells, the plasma membranes are able to come in contact and fuse, comingling the cytoplasms of the two cells. As the opening between the cell walls increases in size, the two nuclei, which travel to the fusion zone are able to fuse, creating a diploid cell¹⁶.

Although many of these processes have been well studied, there are still unanswered questions. We were particularly interested in two aspects of yeast mating. The first, which is described in Chapter 2, centers on the genes required for mating type determination and efficient zygote formation. Much is known about the regulation of mating type from the *MAT* locus¹⁸, but we still lack a complete view of the identification and regulation of the mating type-specific genes activated with and without pheromone induction. We were interested in learning more about what these genes are and how they affect mating efficiency. The second question, which is described in Chapter 3, involves cell wall fusion. Specifically, we were interested in investigating the question of how cells regulate cell wall dissolution spatially and temporally

such that cells dissolve their cell walls only at the point of contact with a mating partner. We hope that by investigating these processes, we can increase the body of knowledge surrounding budding yeast mating – a model for many aspects of cell biology in higher eukaryotes.

OVERVIEW OF THE PHEROMONE RESPONSE PATHWAY

MAP kinase cascade

Intercellular signaling between cells of opposite mating types is important for efficient mating and is one of the more well-studied aspects of mating in budding yeast. Signaling is achieved through seven-transmembrane spanning G-protein coupled receptors, which are expressed on the plasma membrane of each cell and serve to bind the pheromones produced by the opposite mating type^{9,10}. When a G-protein coupled receptor binds the pheromone of the opposite mating type, it goes through a conformational change causing GDP to be exchanged for GTP on Gpa1, the inhibitory α -subunit of the G-protein associated with the pheromone receptors^{19,20}. In its GTP-bound state, Gpa1 does not associate as tightly with the $G_{\beta\gamma}$ subunits (Ste4 and Ste18, respectively), and they are released^{21,22}. Once freed from Gpa1, the farnesyl and palmitoyl groups attached to Ste18, the G_{γ} subunit, associate the $G_{\beta\gamma}$ subunits with the plasma membrane²³, and Ste4 is able to interact with two scaffold proteins, Far1^{ref. 14} and Ste5^{ref. 24}, as well as the p21-activated kinase, Ste20^{ref. 25,26}. Together these three proteins affect the three general pheromone responses: gene transcription^{24,27}, cell cycle arrest¹³, and positive chemotropism¹⁴ (Figure 1.2).

The pheromone-induced transcriptional response is initiated by a MAP kinase cascade⁵, and the $G_{\beta\gamma}$ subunits act, in part, to bring the various parts of the MAP kinase cascade together^{24,26}. The scaffold protein, Ste5, binds the members of the MAP kinase cascade: Ste11, Ste7, and Fus3, bringing them into close physical proximity²⁸⁻³⁰. Ste4 binding to Ste20 and Ste5, in

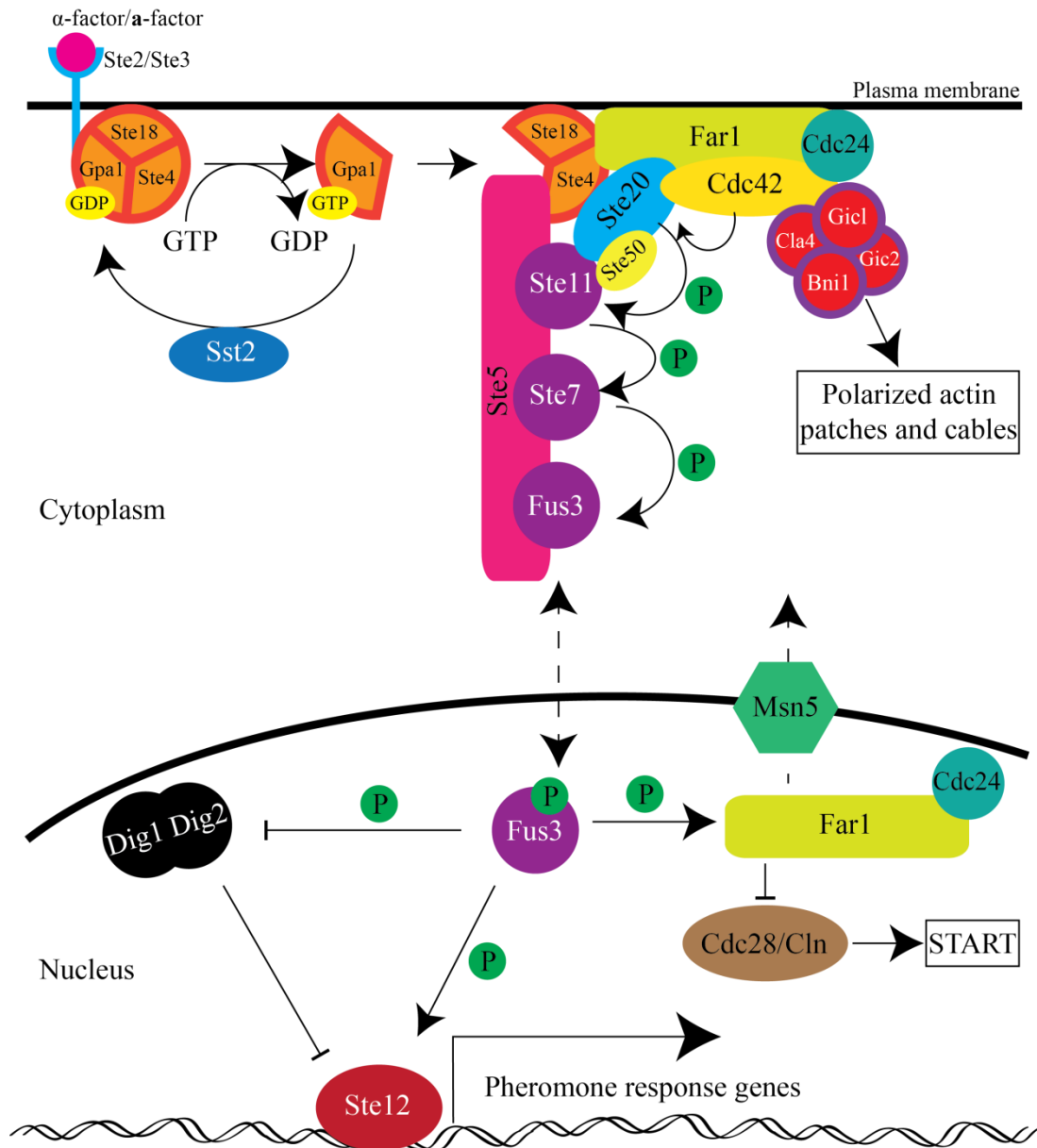


Figure 1.2. Pheromone response pathway. When a pheromone molecule (α -factor or **a**-factor) binds the yeast G-protein coupled receptor (Ste2 or Ste3), GDP is exchanged for GTP on the inhibitory G_{α} subunit (Gpa1) of the G-protein, freeing the $G_{\beta\gamma}$ subunits (Ste4/18) to initiate the pheromone response by binding the two scaffold proteins, Ste5 and Far1, as well as the p21-activated kinase, Ste20. Once in close proximity to Ste11, Ste20, activated by Cdc42, phosphorylates Ste11, which phosphorylates Ste7, which phosphorylates Fus3, causing Fus3 to translocate into the nucleus where Fus3 phosphorylates several targets, including Dig1/2, Ste12, and Far1. Phosphorylated Dig1/Dig2 free the activated Ste12 to induce transcription of pheromone-response genes. Phosphorylated Far1 inhibits the cyclin-dependent kinase (Cdc28/Cln) from initiating START, thus, causing cell cycle arrest. Far1 bound to Cdc24 also translocates out of the nucleus through Msn5. Cytoplasmic Far1 binds to the $G_{\beta\gamma}$ subunits, and Cdc24 activates Cdc42, which, in conjunction with several other proteins, activates polarization of the cytoskeleton in the direction of highest pheromone concentration.

conjunction with the binding of Ste20 and Ste11 by the adapter protein Ste50^{ref. 31-33}, brings Ste20, which is activated by the small rho-like GTPase Cdc42^{ref. 34, 35}, physically close enough to facilitate phosphorylation of Ste11 by Ste20 and initiates the MAP kinase cascade^{26, 27}. Once Ste11 has been phosphorylated, it phosphorylates Ste7^{ref. 36}, which in turn phosphorylates Fus3^{ref. 37, 38}.

Fus3 is able to shuttle in and out of the nucleus, and during pheromone stimulation it can be found near the shmoo tip in complexes with Ste5 and the rest of the MAP kinase cascade to be activated via phosphorylation by Ste7 or in the nucleus³⁹⁻⁴¹, where it binds several targets. To activate the pheromone-induced transcriptional response, phosphorylated Fus3 enters the nucleus and binds to and phosphorylates the proteins Dig1 and Dig2, which act to repress the transcription factor Ste12 in vegetatively growing cells^{42, 43}. Upon Fus3 binding and phosphorylation, Dig1 and Dig2 are inactivated, freeing Ste12^{ref. 42}, which in combination with phosphorylation of Ste12 by Fus3^{ref. 44}, activates Ste12 to initiate transcription of the pheromone-response genes⁴⁵. In the nucleus, activated Fus3 also phosphorylates another scaffold protein, Far1^{ref. 44}, which initiates additional aspects of the pheromone response^{14, 46}.

G1 cell cycle arrest

Activated Far1 is involved with pheromone-induced cell cycle arrest in G1^{13, 46, 47}. Cell cycle arrest is important for efficient mating⁴⁸ so that when nuclear fusion occurs the two nuclei are in the same stage of the cell cycle. Along with its role as a scaffold protein, Far1 also acts as a cyclin-dependent kinase inhibitor through its interactions with the Cdc28/Cln protein complex⁴⁷. When phosphorylated by Fus3, Far1 inhibits the kinase activity of the Cdc28/Cln protein complex⁴⁹. This reduces the ability of Cln1/2/3 to phosphorylate targets required for

progression into S phase⁴⁹ and the ability of Cln3 to promote the transcription of G1-specific gene targets⁵⁰.

Polarization and shmoo formation

The karyopherin, Msn5, facilitates Far1 transport from the nucleus to the cytoplasm⁵¹ where Far1 activates polarization in the direction of highest pheromone concentration by acting as a scaffold protein to bring the polarization machinery to the site of pheromone stimulation¹⁴. In response to pheromone, Far1, bound to the guanine nucleotide exchange factor Cdc24^{ref. 52, 53}, exits the nucleus⁵⁴. Once in the cytoplasm, the Far1-Cdc24 complex binds to Ste4^{ref. 55}, putting Cdc24 in close proximity to the small rho-like GTPase Cdc42^{ref. 14, 34}, which is involved in maintaining cell polarity³⁴ and also binds to Ste20^{ref. 56, 57}. Active Cdc42 bound to the activated G $\beta\gamma$ subunits through Ste20 moves the orientation of the cell from the site of the most recent bud scar to the direction of highest pheromone concentration⁵⁸. Aside from its role in cell polarity, Cdc42 also helps to activate the kinase activity of Ste20 by binding to the inhibitory CRIB domain of Ste20, enabling activation of the kinase domain³⁵, and, thus, phosphorylation of Ste11^{ref. 27}.

Once cells have established a new site of polarity, Cdc42, together with Cla4^{ref. 59}, Bni1^{ref. 60, 61}, and Gic1 and Gic2^{ref. 62}, signals to reorganize the cytoskeleton. The actions of these and additional proteins cause formation of actin patches⁶³ and actin cables⁶¹ directed towards the site of receptor stimulation, which also polarizes secretion in this direction⁶⁴. Polarization of secretion is important for shmoo formation because secretory vesicles carry the additional plasma membrane and cell wall remodeling enzymes needed to increase the size of the plasma membrane and cell wall at the location of polarization^{17, 65, 66}. Additionally, it is possible that

Cdc42 is involved in regulating docking and fusion of these vesicles with the plasma membrane^{67, 68}.

The polarization of the actin cytoskeleton is also important for the localization of pheromone receptors at the point of highest pheromone concentration⁶⁹, which acts to generate positive feedback for polarization towards a strong pheromone source. Polarization of the cell in the direction of highest pheromone concentration is important for efficient mating, enabling the cell to find and choose a mating partner⁷⁰, and cells are able to polarize in the direction of highest pheromone concentration with a high degree of accuracy^{15, 71}. Once two cells have identified each other as possible mating partners, polarization towards the point of contact ensures that the necessary fusion machinery is present enabling cells to fuse to form a single diploid⁷²⁻⁷⁴.

Attenuation of the pheromone response

It is important for efficient mating that cells are capable of attenuating the pheromone response^{75, 76}. There are several mechanisms for this. One protein involved is the GTPase activating protein Sst2^{ref. 75, 76}, which serves to activate the GTP hydrolyzing activity of Gpa1^{ref. 77, 78}. This is important both for inactivation of the pheromone response as well as to stop pheromone independent signaling through the pheromone response pathway^{75, 79}. Free Gpa1 has also been shown to have a role in attenuating the pheromone response through interaction with the MAP kinase Fus3^{ref. 80}. A third protein involved in reducing the sensitivity to pheromone induction in *MATa* cells only is Bar1, the α -factor protease^{75, 76, 81, 82}. By decreasing the concentration of α -factor surrounding the *MATa* cell, Bar1 can reduce the response to pheromone induction in *MATa* cells⁸².

CELL FUSION

Once cells have successfully induced expression of the pheromone response genes, arrested the cell cycle in G1, and polarized towards a potential partner, the two cells must fuse to become a single diploid. The process by which this occurs has four distinct steps: agglutination or attachment of the two cells at their shmoo tips, cell wall fusion, plasma membrane fusion, and nuclear fusion.

Agglutination

Agglutination is the process by which two stimulated cells attach at their shmoo tips, and is accomplished by the action of four cell wall proteins: Sag1^{ref. 83, 84}, Aga1^{ref. 85}, Aga2^{ref. 86}, and Fig2^{ref. 87}. Sag1 is a cell wall protein with sequence similarities to immunoglobulins⁸⁸ that is expressed only in *MATa* cells^{83, 84}. The *MATa*-specific protein, Aga2, is the ligand for Sag1^{ref. 86},⁸⁹, and the interaction of these two proteins is extremely strong, with a K_D on the order of $10^{-9}M$ ⁹⁰. Aga2 does not attach directly to the cell wall, but rather attaches via disulfide bonds to the haploid-specific glycosylphosphatidylinositol (GPI)-anchored protein, Aga1^{ref. 85, 91}. Deleting any of these proteins has a small to insignificant effect on mating efficiency on solid media, but a significant effect in liquid media^{83-85, 92}.

The role of Fig2, a protein expressed in both *MATa* and *MAT α* cells⁸⁷, in mating is somewhat more complex. Along with its role in agglutination, Fig2 is also involved in accurate detection of pheromone gradients, shmoo formation, cell fusion⁸⁷, and pheromone-induced invasive growth⁹³. Additionally, cells deleted for *FIG2* show decreased viability when pheromone stimulated and during mating, particularly when the osmolarity of the extracellular environment is decreased⁹⁴. In contrast to simple agglutinins, *fig2 Δ* cells show an increased mating efficiency in liquid media and a slightly decreased mating efficiency on solid media⁸⁷. In its role as a mating agglutinin, the presence of Fig2 seems to be able to compensate for mutations

in the other three mating agglutinins⁹³. However, deleting all of the mating agglutinins in given cell type causes a significant mating defect even on solid media⁹³.

Cell Wall Fusion

Even after cells have successfully attached by their shmoo tips via mating agglutinins, their plasma membranes are still separated by their cell walls, a distance greater than 200nm⁹⁵. To proceed, the cell wall that lies between the two membranes must be dissolved, and the boundaries of the remaining cell walls, which surround the site of cell fusion, must fuse to form a single, continuous structure that will surround the newly formed zygote¹⁶. This spatially regulated cell wall dissolution and fusion is a dangerous task because of the osmotic pressure differential between the cytoplasm and the extracellular environment^{96,97}. If the cell wall is opened at the wrong time or in an inappropriate location, exposing the plasma membrane directly to the environment, there will be no elastic force to resist the turgor pressure of the cell, water will rush into the cell from the extracellular environment, and the cell will lyse^{96,97}.

Because of the inherent dangers in this process it seems reasonable to assume that it is tightly regulated, and a significant amount of work has been done to investigate this. Mutations in genes involving cell fusion tend not to cause sterility, particularly when there is one wildtype partner, making screening for them difficult. However, a significant number of proteins have been implicated in cell wall fusion (Figure 1.3). One of these is the membrane-spanning protein, Fus1^{ref. 98, 99}. Fus1 is localized to the shmoo tip during pheromone-stimulation⁹⁸ by the exomer complex component, Chs5^{ref. 100, 101} and lipid rafts at the plasma membrane⁷², and has been hypothesized to act as a scaffold protein that stabilizes the localization of fusion machinery at the fusion zone¹⁰².

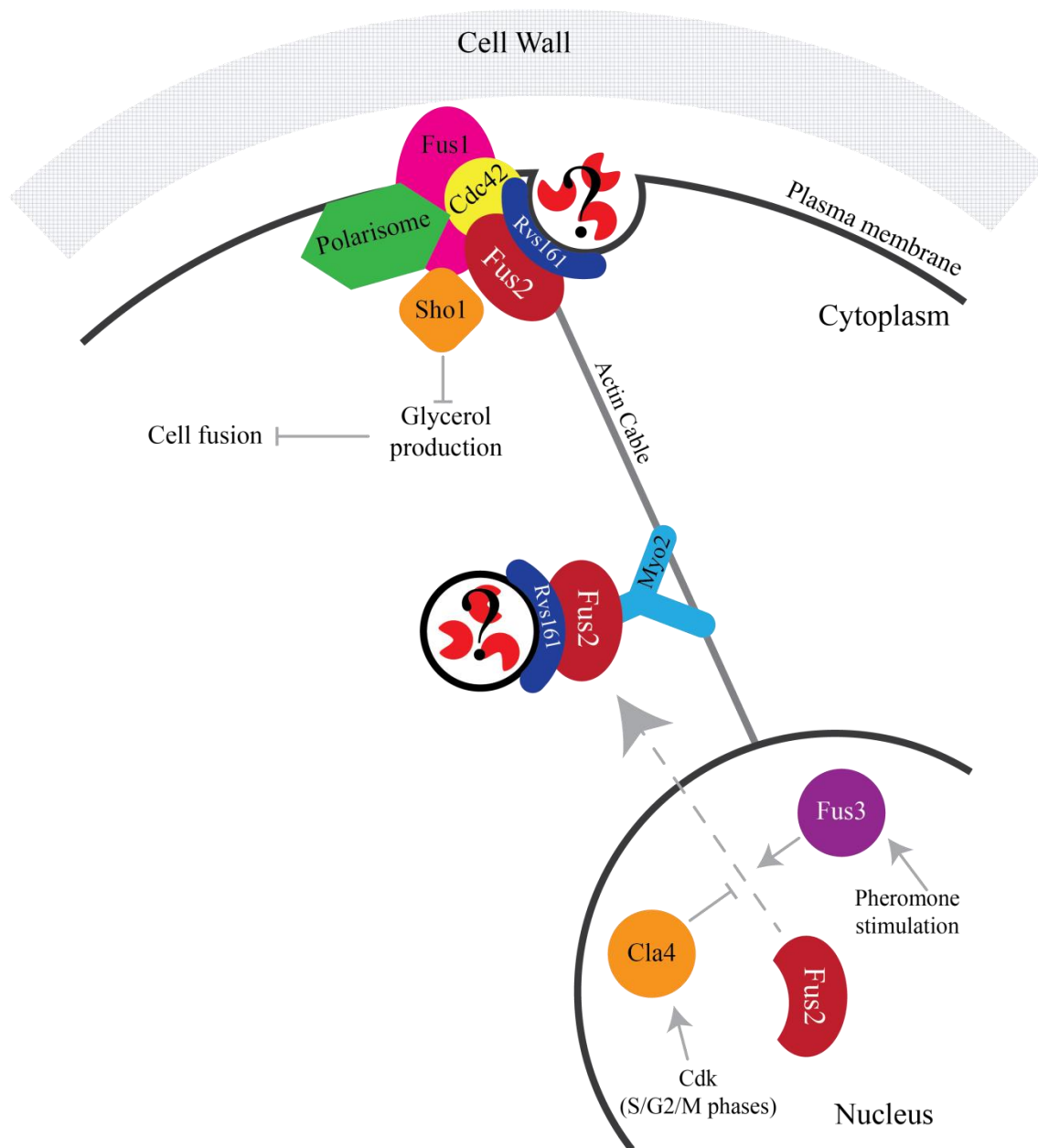


Figure 1.3. Cell wall fusion. During the pheromone response, the cellular localization of Fus2 is dependent on Cla4, which localizes Fus2 to the nucleus in S, G2, and M phases, and Fus3, which activates cytoplasmic localization of Fus2 in response to pheromone. Cytoplasmic Fus2 associates with Rvs161, which may bind secretory vesicles. Fus2 travels along actin cables in a Myo2-dependent fashion, presumably with Rvs161 and secretory vesicles, to the shmoo tip, where Fus2 binds the scaffold protein Fus1, and together with GTP-bound Cdc42, is hypothesized to stimulate the secretory vesicles to release their cargo (believed to be cell wall remodeling enzymes) into the cell wall. Fus1 also binds the polarisome, which is necessary for tight clustering of the secretory vesicles, and Sho1, which inhibits glycerol production to allow for cell wall fusion.

Efficient fusion requires high levels of pheromone production⁶⁶, osmotic regulation⁹⁶, and polarization of vesicles at the fusion zone^{66, 103}. Although Fus1 is not known to be necessary for pheromone production and secretion, it is involved in both osmotic regulation¹⁰² and polarization of vesicles at the fusion zone¹⁰³.

Cells that cannot regulate the amount of internal glycerol present in their cytoplasm have cell fusion defects, implicating the high-osmolarity glycerol response as a regulator of cell fusion⁹⁶. Through an interaction with the osmosensor, Sho1^{ref. 104}, Fus1 negatively regulates glycerol production and promotes cell fusion¹⁰². There are several reasons that the regulation of glycerol production might be important during mating. One is that cells whose internal osmolarity more closely matches that of the extracellular environment may be able to proceed through cell wall fusion in a safer fashion. Another possibility is that cells need to reduce the internal osmotic pressure in order to allow the two shmoo tips to flatten and make a larger region of cell wall apposition, which could act both to increase the safety of cell wall fusion and to increase the concentration of secreted cell wall remodeling enzymes that build up in this region by increasing the diffusional path length these enzymes must travel prior to exiting the cell wall.

When prezygotes are observed using electron microscopy, it is possible to see a large number of vesicles tightly clustered around the fusion zone⁶⁶. In *fus1* mutants, the vesicles are largely absent, indicating that Fus1 is involved with localization of these vesicles to the fusion zone¹⁰³. When mutations are made in *SPA2*, a member of the polarisome¹⁰⁵ that is involved in cell fusion¹⁰⁶, the vesicles are present but dispersed, indicating that Spa2, and perhaps the entire polarisome, is involved in vesicle clustering¹⁰³. In the double *fus1 spa2* mutant, vesicles are largely absent from the fusion zone and the ones that are there are dispersed¹⁰³. Fus1 also interacts with Bni1^{ref. 102}, another member of the polarisome¹⁰⁵ involved in cell fusion¹⁰⁶.

The tight polarization of vesicles at the fusion zone is a complex and highly regulated process. During pheromone stimulation, transcription of pheromone-induced genes can occur in any stage of the cell cycle^{107, 108}. However, it is important that cell fusion only occur during G1 so that the ploidy of the nuclei is the same. To at least partially control this, the localization of Fus2, another protein involved in cell wall fusion⁹⁸, is tightly regulated^{107, 108}. In mitotic cells, Fus2 is sequestered in the nucleus¹⁰⁸, which is controlled via phosphorylation by Cla4 in a cyclin-dependent kinase-dependent manner¹⁰⁷. When pheromone-stimulated cells arrest in G1, Fus3 phosphorylates Fus2, enabling it to exit the nucleus¹⁰⁸.

Once cytoplasmic, Fus2 is free to associate with Rvs161^{ref. 109, 110}, a protein that binds to curved membranes^{111, 112}, such as those of vesicles, and plays a role in endocytosis¹¹³ and cell fusion¹¹⁴. To localize to the fusion zone, Fus2 travels along actin cables in a type V myosin motor (Myo2)-dependent fashion¹¹⁵, presumably while associated with Rvs161 and the vesicles seen localized to fusion zone¹¹⁶. Fus1 anchors Fus2 at the fusion zone¹¹⁶, and they bind GTP-bound Cdc42^{ref. 102}, which together with its guanine nucleotide exchange factor, Cdc24, has a role in cell fusion separate from its role in either cell polarization or in the MAP kinase signaling pathway¹¹⁷⁻¹¹⁹. The process by which the vesicles fuse with the plasma membrane is still unclear. However, it has been hypothesized that Fus2 and Rvs161 in conjunction with Cdc42 stimulate fusion of the vesicles with the plasma membrane and release of the contents into the extracellular space^{116, 119}. The contents of the fusion vesicles are not known, but it seems reasonable to assume that they are cell wall remodeling enzymes involved in cell wall dissolution and fusion. A decrease in β -1-3-glucan in the cell wall at the shmoo tip appears to be a prerequisite for fusion, and a negative regulator of β -1-3-glucan synthesis, Lrg1, is necessary for efficient fusion¹²⁰. Three putative glucanases, Scw4, Scw10, and Scw11, have also been implicated in mating^{121, 122}

and may be among the enzymes packaged into the fusion secretory vesicles as cells lacking both Scw4 and Scw10 have decreased mating efficiency in crosses with genetically identical partners¹²¹.

It is easy to understand why limiting cell wall fusion to moments in G1 when the osmolarity of the cell is low enough to prevent lysis and the pheromone concentration is high is beneficial. However, prior to fusion the cell wall must be dissolved, and none of the elucidated regulations of cell wall fusion limit this dissolution spatially and temporally to the site of fusion with a polarized partner. In Chapter 3, I will present evidence for a mechanism by which cell wall dissolution is limited to the fusion zone with a mating partner by a contact-driven increase in the concentration of cell wall remodeling enzymes.

Plasma membrane fusion

The next step in cell fusion, plasma membrane fusion, is another dangerous process that begins as soon as a pore has formed between the two cell walls¹⁰³. Although the prezygote is now safely incased in a cell wall, which protects it from the dangers of osmotic lysis, it must still fuse the plasma membrane, which separates the inside of the cell from the external environment. The plasma membrane consists of a lipid bilayer, and both leaflets of the bilayer must be fused to achieve cytoplasmic mixing (Figure 1.4). For this to safely occur the outer leaflets of the two plasma membranes fuse first, creating a hemifusion stalk. At this point the outer leaflet of the two cells is a continuous structure, but the inner leaflets are unfused, so the cytoplasms of the two cells are still separate. Over time, the hemifusion stalk can either expand until the curvature of the two inner leaflets is such that it is energetically favorable for the two inner leaflets to fuse, forming a fusion pore, or dissociate, leaving the two plasma membranes unattached¹²³. The formation of hemifusion stalks and subsequent formation of fusion pores requires specialized

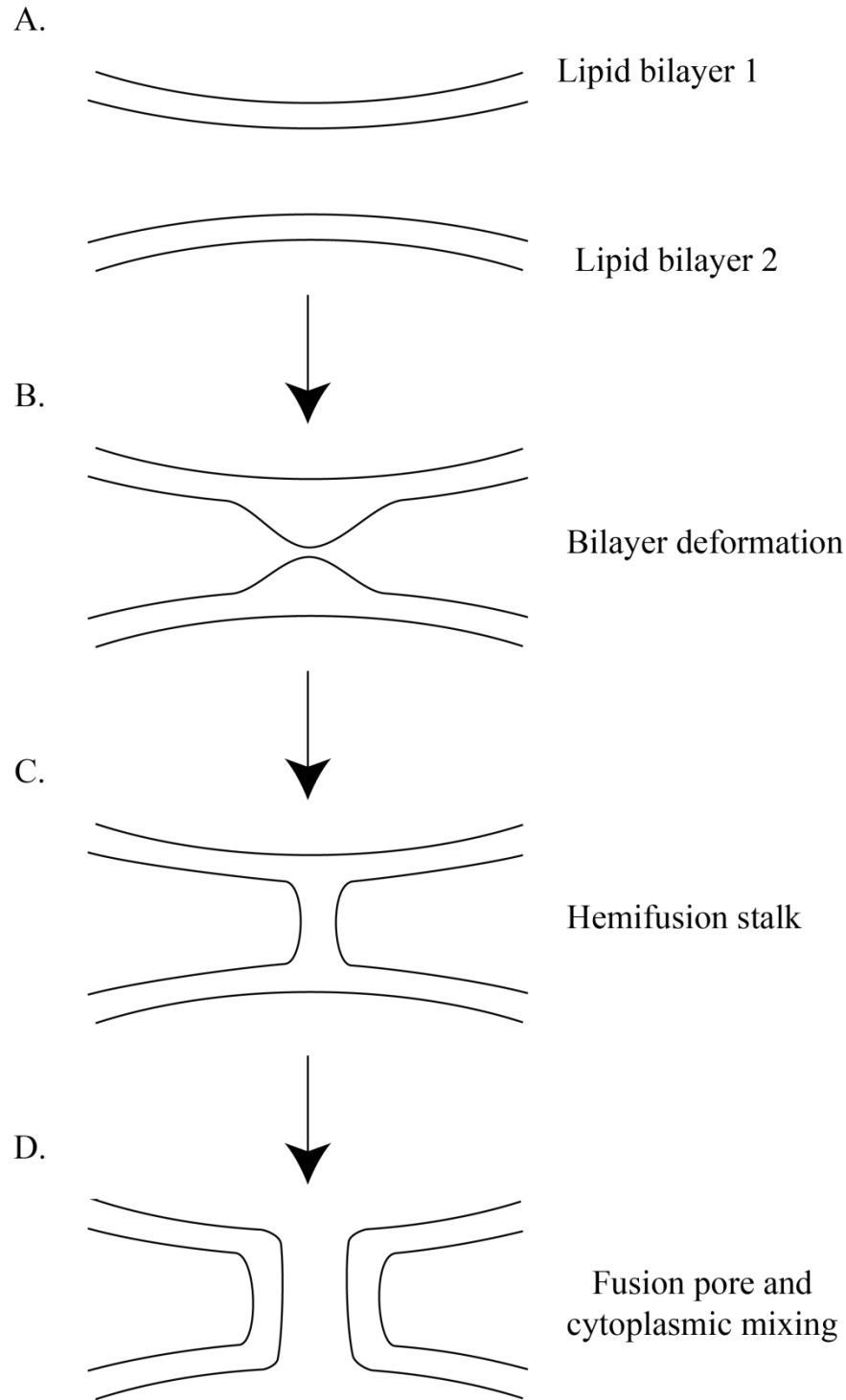


Figure 1.4. Plasma membrane fusion. **A.** After cell wall fusion, the two plasma membranes appose each other. **B.** Plasma membrane fusion is energetically unfavorable, so the lipid bilayers are thought to first deform to decrease the energetic barrier to fusion. **C.** First the outer leaflets of the plasma membrane fuse, causing formation of a hemifusion stalk. **D.** Then the inner leaflets of the plasma membranes fuse, causing formation of a fusion pore and enabling cytoplasmic mixing. Proteins, such as Prm1 and Fig1, may help in any one of these steps.

conditions to make the process energetically favorable, which include membrane curvature, the relative lipid content of the membranes, and stabilizing the fusion intermediates¹²⁴. When these processes are not carefully controlled, instead of creating a fusion pore, holes in the plasma membranes that leave the cytoplasms open to the extracellular environment can form. To safely achieve plasma membrane fusion, it is thought that membrane proteins, conceptually analogous to the SNAREs involved with the fusion of secretory vesicles, are required¹²⁵. To this end, several proteins have been implicated in plasma membrane fusion.

The first protein identified to play a role in membrane fusion is Prm1^{ref. 74}, a pheromone stimulated membrane protein with four transmembrane domains¹²⁶ that localizes to the fusion zone during mating⁷⁴. When *PRM1* is deleted from both mating partners, approximately half of the prezygotes stall after cell wall dissolution with the two plasma membranes about 8nm apart⁷⁴. Several years later, Fig1, a protein involved in the low affinity Ca^{2+} influx system¹²⁷, was also shown to be involved in plasma membrane fusion¹²⁸. Prm1 and Fig1 appear to stabilize fusion pores. When they are absent from mating pairs, prezygote lysis occurs in a Ca^{2+} -dependent manner, potentially because of the role for Ca^{2+} in membrane wound repair¹²⁸⁻¹³¹. There are several hypotheses for how Prm1 and Fig1 might go about stabilizing fusion pores. One is that they act to hold the two apposing membranes together, which is supported by the presence of a hydrophobic loop on Prm1 that is not transmembrane^{126, 128}. Another possibility is that the two proteins, perhaps aided by oligomerization of Prm1 via disulfide bonds, surround the fusion pore either to hold an as yet undiscovered fusase in place or to corral the lipids in the fusion pore to stabilize the pore^{126, 132}. Work on how this occurs and which model is correct is still ongoing.

Several other proteins have been implicated in plasma membrane fusion. One of these is Kex2¹³³, a Golgi-resident¹³⁴ Ca^{+2} -dependent protease involved in the maturation of many

proteins¹³⁵⁻¹³⁷, including α -factor¹³⁸. The mechanism through which Kex2 promotes plasma membrane fusion is unclear, but it is likely through modifications of a substrate(s) necessary for efficient membrane fusion¹³³. Ergosterol in the plasma membrane has also been shown to play a role in membrane fusion¹³⁹, suggesting that it has a role in both fusion and polarization^{72, 139}. Perhaps surprisingly, along with its role in cell wall fusion, Fus1 appears to be involved with efficient plasma membrane fusion pore enlargement after formation¹⁴⁰. Plasma membrane fusion is arguably one of the least well studied of any of the processes described here, and at the moment it is difficult to form a coherent model for how membrane fusion during mating occurs.

Karyogamy

In order for the binucleate cell to form a single diploid nucleus, after plasma membrane fusion, the cell must proceed through nuclear fusion, also termed karyogamy. Nuclear fusion has two distinct parts. The two nuclei must first migrate towards each other, in a process known as nuclear congression, which is accomplished by microtubules that interact with the spindle pole body on each nucleus. Once the two nuclei come in contact with each other, they must go through nuclear envelope fusion¹⁶. Kar4, a transcription factor whose expression is induced by Ste12 in a pheromone-regulated manner, is required to activate transcription of many of the genes involved in karyogamy. This regulates the timing of karyogamy by ensuring that the genes involved are not transcribed until after the first wave of the pheromone response¹⁴¹.

Prior to cell fusion, the nucleus migrates towards the shmoo tip in preparation for fusion via cytoplasmic microtubules emanating from the spindle pole body. The microtubule minus ends attach to the spindle pole body¹⁴². To facilitate nuclear congression, the protein Bim1 attaches to microtubule plus-ends^{143, 144} and forms a complex with Kar9^{ref. 145, 146}. Kar9 is thought to interact with the type V myosin motor protein, Myo2, which pulls the microtubules, and thus

the nucleus, along actin cables to the shmoo tip^{147, 148}. Once at the fusion zone, Bim1 and Kar9 anchor polymerizing plus-ends to the shmoo tip via interactions with Myo2 and the polarisome¹⁴⁹. Two other proteins, Kar3 and Cik1, which together act as a kinesin motor¹⁵⁰, bind the depolymerizing plus-ends of microtubules¹⁵¹ and anchor them to the shmoo tip¹⁵² via interactions with the G_α subunit, Gpa1^{ref. 153}.

After plasma membrane fusion, the two nuclei continue to move towards each other via the action of microtubules and microtubule motor proteins, and there are two models for how this may occur. One possible model is that after pores have formed between the plasma membranes of the two cells, microtubules emanating from each of the nuclei contact each other in a Kar3-dependent fashion. Once the two microtubules are attached, Kar3 together with Cik1 stimulates plus-end depolymerization of the microtubules, shortening them, and effectively pulling the nuclei towards each other¹⁵⁴. A second model is that the microtubules from one nucleus interact with the spindle pole body of the other nucleus in a Kar3-dependent fashion. Force is then generated by motors on the two spindle pole bodies that enables each spindle pole body to slide along microtubules anchored to the other spindle pole body, thus, pulling the two nuclei together¹⁵⁵. These two models are not mutually exclusive; so, it is possible they each explain an aspect of nuclear congression.

Fusing cells that successfully bring their nuclei in close contact are able to begin the last stage of cell fusion: nuclear envelope fusion. The nuclear envelope consists of two concentric membranes and a spindle pole body, each of which must be fused in order to generate a diploid nucleus¹⁵⁶. The molecular mechanisms of nuclear fusion are still being elucidated, but some proteins have been identified to play a role in each phase. The first step in nuclear fusion is fusion of the outer membrane¹⁵⁶. Prm3, a protein which resides on the cytoplasmic side of the

nuclear envelope is involved in this process, perhaps as a fusase or as the facilitator of a fusase¹⁵⁷,¹⁵⁸. Prm3 interacts with Kar5^{ref. 158}, which is involved with both the initiation of outer membrane fusion and the subsequent expansion of the outer membrane fusion pore¹⁵⁷. Sec66, a protein involved in transport into the ER, has also been implicated in outer membrane fusion¹⁵⁹. Additionally, Kar5 might be involved with linking the inner and outer membranes together at the fusion pore, enabling inner membrane fusion at the fusion pore¹⁵⁷. To this end, Kar5 may form a complex with the DnaJ-like chaperone protein, Jem1^{ref. 160}, and the ATPase, Kar2^{ref. 161}, which are necessary for the next step: inner membrane fusion^{157, 159, 162}. The last step in the process is fusion of the spindle pole body¹⁵⁶. The proteins involved in this step are not clear, but several have been put forward as possible players, including Jem1 and Mps3^{ref. 159, 163}, a protein involved in insertion of the spindle pole body into the nuclear envelope after mitotic division¹⁶⁴. Mps3 has also been hypothesized to play a role in working with Kar5 to hold the inner and outer membranes together for coordinated fusion of the nuclear envelope¹⁵⁷.

Following successful formation of a diploid nucleus, the newly formed zygote reenters into the mitotic cycle as a diploid. The methods by which it does this are not clear, but it likely involves the proteins mentioned previously as attenuators of the pheromone response, Bar1 and Sst2^{ref. 75, 76} as well as the *MATa*-specific protein, Asg7, which together with the **a**-factor receptor, Ste3, causes internalization of the G_β subunit of the G-protein^{165, 166} and will be discussed in a subsequent section of this review. Aside from Ste3's interaction with Asg7, there are currently no *MATa*-specific proteins known to be involved in attenuation of signaling through the pheromone-response pathway¹⁶⁷. However, in Chapter 2 I will discuss the identification of a *MATa*-specific **a**-factor barrier protein that would attenuate signals through Ste3 and the

observation that cells that are *MAT α* at the *MAT* locus but express Ste2 instead of Ste3 only arrest transiently during pheromone stimulation.

MATING TYPE DETERMINATION

MAT locus

An important feature of the budding yeast cell cycle and mating in particular is the presence of three different cell types: two haploid mating types – *MAT \mathbf{a}* and *MAT α* and diploid cells – *MAT \mathbf{a}/α* . The cell fate of a budding yeast cell is controlled at the *MAT* locus on Chromosome III (Figure 1.5). Each cell type expresses a different combination of transcription factors at the *MAT* locus, and in combination with several constitutively expressed transcription factors these control the expression of a wide variety of genes^{18, 167}.

Expression of *MAT \mathbf{a}* -specific genes is the default state of cells lacking expression of any transcription factors at the *MAT* locus¹⁶⁸. Because of this, the two open reading frames present in the *MAT \mathbf{a}* locus, *MAT \mathbf{a} 1* and *MAT \mathbf{a} 2*, do not have any known function in haploid cells¹⁶⁹. The *MAT α* locus also has two open reading frames, which encode the genes *MAT α 1* and *MAT α 2*^{ref. 170}. *MAT α 1* acts as a transcriptional activator to turn on expression of α -specific genes, while *MAT α 2* acts as a transcriptional repressor to turn off expression of \mathbf{a} -specific genes¹⁶⁸. In cells expressing both the *MAT α* and *MAT \mathbf{a}* loci, such as diploids, *MAT α 2* and *MAT \mathbf{a} 1* form a complex, which turns off expression of all haploid-specific genes¹⁷¹. Because only cells expressing transcription factors from a single *MAT* locus can mate, I will focus on these cell types in this review.

Cell type specific gene expression

The control of mating type from the *MAT* locus has been extremely well studied. However, determining all of the genes expressed in each of the mating types is a more difficult

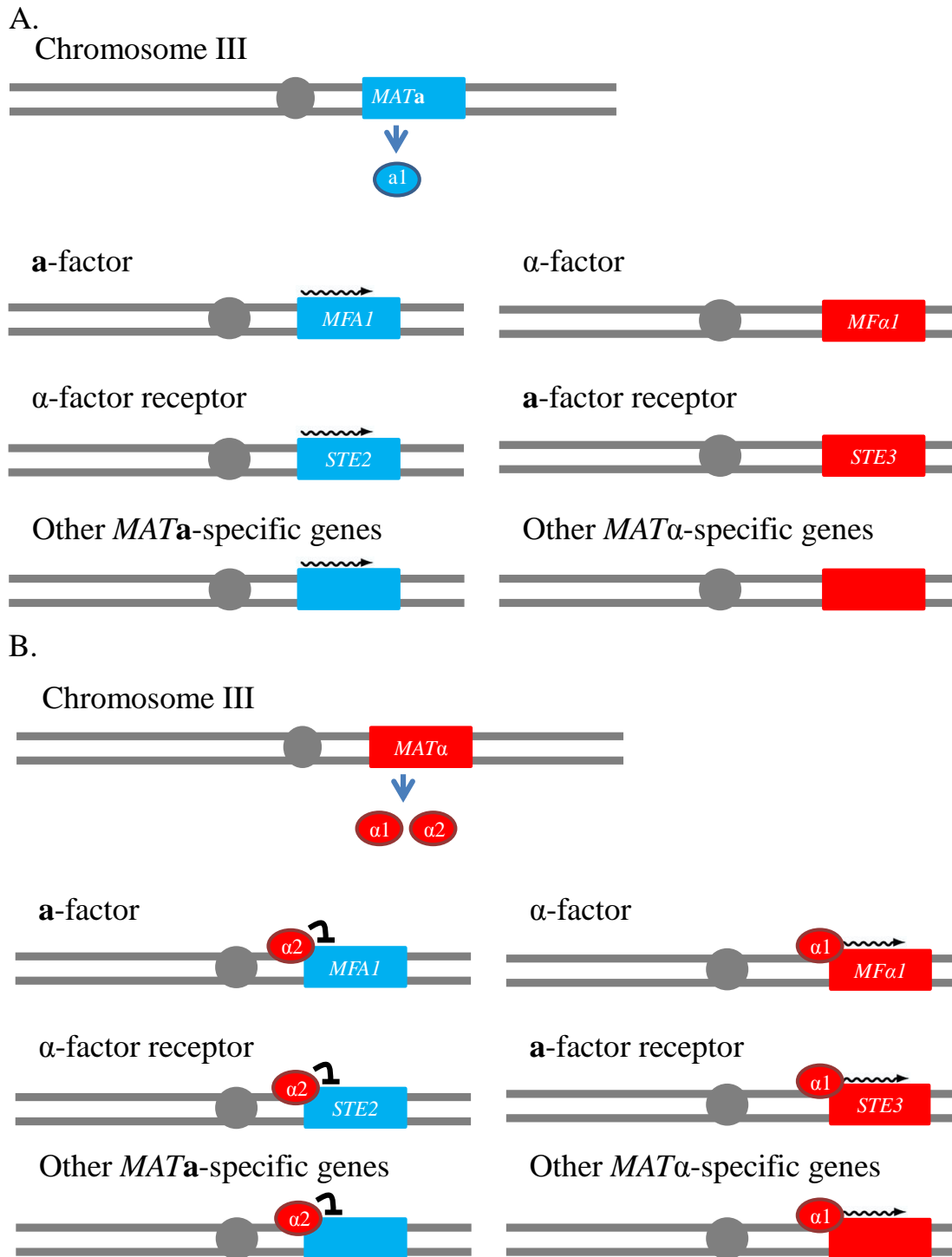


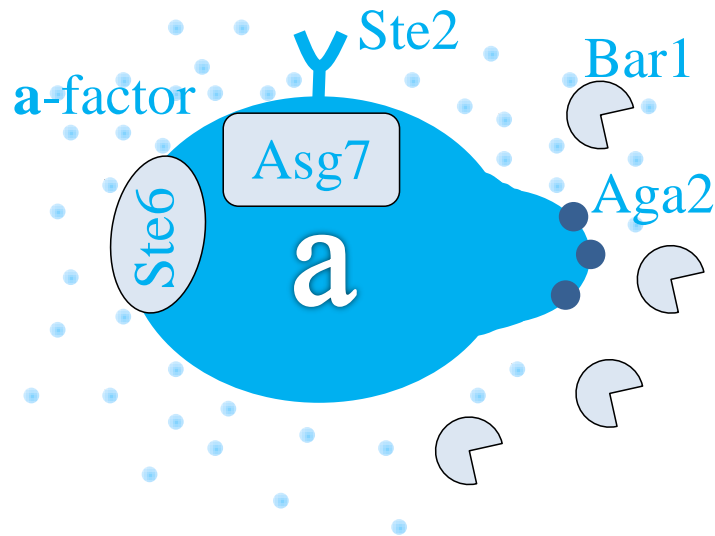
Figure 1.5. Control of haploid mating type by the *MAT* locus. **A.** *MATa*-specific genes are repressible, so the transcription factor expressed at the *MATa* locus, *Mata1*, has no known function in haploid cells. Cells that are *MATa* at the *MAT* locus express *MATa*-specific genes and do not express *MAT α* -specific genes. **B.** The *MAT α* locus expresses two transcription factors, *Mata1* and *Mata2*. *Mata1* activates transcription of *MAT α* -specific genes. *Mata2* represses transcription of *MATa*-specific genes.

task. Prior to the advent of high throughput techniques such as microarrays, RNA sequencing, and chromatin immunoprecipitation, in order to screen for a gene, a specific function needed to be identified, so the identification of mating type-specific genes was generally limited to functions such as mating for *MATa* and *MATα* cells or meiosis for *MATa/α* cells. In 2004, Galgoczy, *et al.* did a genome-wide study of the three different cell types and assembled a more complete list of *MATa*-, *MATα*-, and *MATa/α*-specific genes based on *Mata1*, *Mata2*, and *Mata1/Mata2* binding sites. However, these experiments were done in the absence of pheromone and, while they did identify sites of *MAT* transcription factor binding, they did not probe the potential intricacies of the differences between pheromone induction in *MATa* and *MATα* cells¹⁶⁷. Genome-wide studies of the transcriptome of pheromone-stimulated haploid cells have been done¹⁷². Although the analysis of α -factor stimulated *MATa* cells was fairly straight forward, comparing this data set to that of *a*-factor-stimulated *MATα* cells at similar levels of pheromone induction is extremely complex due to the hydrophobicity of *a*-factor¹⁷². In Chapter 2, I describe a system that allows for controllable analysis of the transcriptome of pheromone-stimulated *MATα* cells.

MATa cells

Aside from the transcription factors expressed at the *MATa* locus and genes involved in mating type switching, a process eliminated from most domesticated laboratory strains, there are 7 *MATa* specific genes: *STE2*, *MFA1*, *MFA2*, *STE6*, *BARI*, *AGA2*, and *ASG7*, all of which function in mating¹⁶⁷ (Figure 1.6A). As previously mentioned, *STE2* encodes for a seven-transmembrane G-protein coupled receptor that binds to α -factor¹¹ and activates the MAP kinase cascade⁵ while *MFA1* and *MFA2* encode for the pheromone *a*-factor^{6, 173}. Each of these *a*-factor genes is responsible for approximately half of the *a*-factor production¹⁷³. Deletion of a single *a*-

A.



B.

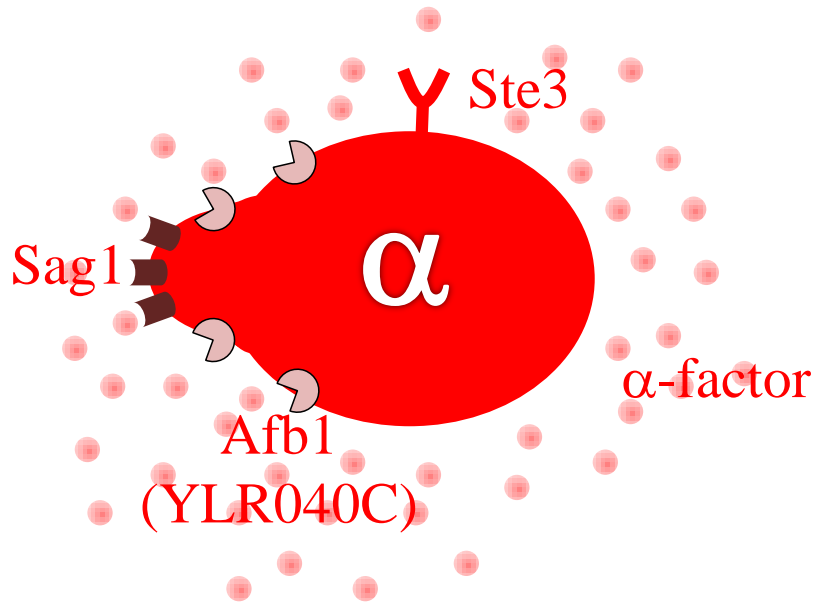


Figure 1.6. *MATa*- and *MATα*-specific genes. **A.** *MATa* cells express the pheromone, **a**-factor, and the α -factor receptor, Ste2. **a**-factor is secreted through the ABC transporter, Ste6. *MATa* cells also express the α -factor protease, Bar1, and the **a**-agglutinin, Aga2. **B.** *MATα* cells express the pheromone, α -factor, and the **a**-factor receptor, Ste3. They also express the α -agglutinin, Sag1, and the uncharacterized ORF *YLR040C*, which we will demonstrate encodes the novel **a**-factor barrier protein, Afb1.

factor gene has little effect on mating efficiency, but deletion of both *MFA1* and *MFA2* causes sterility in *MATa* cells¹⁷³. Because of the extreme hydrophobicity of **a**-factor, it cannot exit the cell via the secretory pathway. Instead *STE6* encodes a plasma membrane ATP-binding cassette transporter that transports mature **a**-factor out of the cell¹⁷⁴. Although the other 3 genes encode proteins that make mating more efficient, *Ste2*, **a**-factor, and *Ste6* are the only *MATa*-specific proteins that are essential for zygote formation¹⁷⁵.

Bar1 is an aspartyl protease that is secreted by *MATa* cells to cleave α -factor^{81, 176}, and is important for efficient mating⁷⁰. As would be expected, cells lacking *Bar1* are more sensitive to α -factor arrest^{75, 76}, but perhaps paradoxically, these cells also have a more difficult time finding a mating partner^{70, 177}. This can be explained by the fact that α -factor diffuses radially from each *MATa* cell, so in a dense mating mixture the direction of highest pheromone concentration could be an average of many α -factor producing points as opposed to in the direction of a nearby mating partner. *Bar1* that exits the cell wall and diffuses away from the *MATa* cell decreases the distance free α -factor peptides travel prior to degradation, thus decreasing the number of α -factor producing points that any individual *MATa* cell senses and privileging the α -factor secreted from nearby cells¹⁷⁷. Complementarily, *Bar1* that is trapped in the cell wall of the *MATa* cell creates an α -factor sink, which helps the *MATa* cell to distinguish between two close, potential *MATa* partners such as might be found in an ascus after sporulation or in a dense mating mixture by keeping the α -factor concentration low enough that every pheromone receptor on the surface of the *MATa* cells is not bound to α -factor¹⁷⁸. The α -factor sink surrounding each *MATa* cell also increases the chance that each *MATa* cell will chose a different *MATa* partner, since *MATa* cells are unlikely to polarize towards the sink of a nearby *MATa* neighbor and will tend to avoid each other as they polarize¹⁷⁹.

As discussed previously, once cells have accurately detected a gradient, shmooed towards each other, and come in contact, the mating agglutinins attach the two cells at the shmoo tip¹⁶. The **a**-agglutinin is a complex of two proteins, Aga1^{ref. 85} and Aga2^{ref. 86}. Aga1 is not mating type specific and is necessary for cell surface attachment of Aga2^{ref. 85}. Aga2, which is *MATa*-specific is the ligand for the α -agglutinin^{86,89}. The *MATa* mating agglutinins are dispensable when cells mate on solid media but are necessary when cells mate in liquid⁸⁵.

The final *MATa*-specific protein is Asg7, which is important for overcoming pheromone-stimulation in early zygotes¹⁶⁶. Immediately after two haploid cells fuse, they will express all of the *MATa*-specific proteins and the *MAT α* -specific proteins, including the pheromones and pheromone receptors. It is possible to imagine that due to both autocrine signaling and the pheromones present in a dense mating mixture, the pheromone receptors would continue to bind ligand, which would keep the newly fused zygote in G1 cell cycle arrest until the pheromone receptors could be endocytosed. When Asg7, which is only expressed in *MATa* cells¹⁸⁰, comes in contact with Ste3, which is only expressed in *MAT α* cells¹⁰, in the early diploid, Asg7 likely binds to Ste4, the G β subunit, causing Ste4 to be internalized¹⁶⁵. Since internalized Ste4 can no longer recruit Ste5 to the plasma membrane, signaling through the MAP kinase cascade is decreased¹⁶⁵, allowing early diploids to proceed through mitosis¹⁶⁶.

***MATa* cells**

There are 5 known *MAT α* -specific genes: *STE3*, *MFa1*, *MFa2*, *SAG1*, and *YLR040C*^{ref. 167} (Figure 1.6B). As described previously, Ste3 is the seven-transmembrane G-coupled **a**-factor receptor¹⁰ and *MFa1* and *MFa2* encode for the pheromone α -factor^{7,8}. Unlike in *MATa* cells the production of α -factor from *MFa1* and *MFa2* is not distributed equally between the two genes¹⁸¹. The majority of α -factor production occurs from *MFa1* and deletion of this gene causes a 75%

decrease in mating efficiency¹⁸¹. *MFa2* produces much less α -factor, and the effect of its deletion on mating efficiency is correspondingly lower¹⁸¹. α -factor is secreted through the normal secretory pathway, and does not necessitate a dedicated α -factor secretion mechanism⁷. As in *MATa* cells, these are the only *MATa*-specific proteins required for zygote formation¹⁷⁵.

As mentioned earlier, *MATa* cells also express the α -agglutinin, Sag1^{ref. 83}, a GPI-anchored protein expressed in the cell wall⁸⁴. The N-terminal half of the protein has sequence similarities to immunoglobulins⁸⁸, and the **a**-agglutinin, Aga2, acts as the ligand for Sag1^{ref. 86, 89}. Sag1 is only necessary for mating in liquid media^{83, 84}.

It seems reasonable to hypothesize that *MATa* cells in a dense mating mixture would have similar problems in polarizing in the direction of highest pheromone concentration as *MATa* cells due to the **a**-factor secreted by multiple, potential *MATa* partners. However, although an **a**-factor barrier protein has been purported to exist^{182, 183}, it has not been definitively identified. In Chapter 2, I provide evidence to suggest that the final *MATa*-specific gene, *YLR040C*, encodes for an **a**-factor barrier protein, which we name *AFBI* for **a**-factor **b**arrier. Much of what is known about *AFBI* has been gathered through high throughput experiments about its expression patterns. Briefly, *AFBI* has a Mat α 1 binding site that controls its expression¹⁶⁷, has been shown to be translated by ribosome profiling¹⁸⁴, and localizes to the cell wall^{185, 186}. This serves to show that although the body of knowledge surrounding budding yeast haploid cell types is considerable, there is still much to be learned. The experiments I describe in Chapter 2 seek to improve our understanding of the differences in *MATa* and *MATa* cells and the roles of the proteins that embody these differences.

CLOSING REMARKS

The study of budding yeast mating helps bring to light the mechanisms involved in many of the daily cellular functions of higher eukaryotes: from fertilization to cellular differentiation to intercellular signaling to development of cellular polarity. I have given a brief overview of the current state of knowledge about many of the processes involved in budding yeast mating, which highlights both how much we have discovered and how much is left to uncover. Although we know much about the control of the cell types in budding yeast through the transcription factors expressed from the *MAT* locus, we have not determined the role of all of these genes in intercellular signaling and fusion. In Chapter 2, I will discuss experiments to further elucidate the differences between the two haploid cell types in both gene expression and pheromone response as well as to provide a peek into the necessity for careful regulation of the expression of these genes for efficient mating. Once two cells have successfully located each other, they must go through the dangerous process of fusing to form a single zygote. In Chapter 3, I will provide evidence for a hypothesis that addresses the careful spatial and temporal regulation of cell wall fusion necessary to reduce the chance of osmotic lysis. The evidence presented in both of these chapters helps to pave the way for future research, and in Chapter 4 I will discuss the conclusions we can draw and possible future directions of the study of mating in the budding yeast.

References

1. Cross, F., Hartwell, L. H., Jackson, C. & Konopka, J. B. Conjugation in *Saccharomyces cerevisiae*. *Annu. Rev. Cell Biol.* **4**, 429-455 (1988).
2. Herskowitz, I. Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiol. Rev.* **52**, 536-553 (1988).
3. Herskowitz, I. A regulatory hierarchy for cell specialization in yeast. *Nature* **342**, 749-757 (1989).
4. Nasmyth, K. & Shore, D. Transcriptional regulation in the yeast life cycle. *Science* **237**, 1162-1170 (1987).
5. Bardwell, L. A walk-through of the yeast mating pheromone response pathway. *Peptides* **26**, 339-350 (2005).
6. Betz, R. & Duntze, W. Purification and partial characterization of a factor, a mating hormone produced by mating-type-a cells from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **95**, 469-475 (1979).
7. Kurjan, J. & Herskowitz, I. Structure of a yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor. *Cell* **30**, 933-943 (1982).
8. Singh, A. *et al.* *Saccharomyces cerevisiae* contains two discrete genes coding for the alpha-factor pheromone. *Nucleic Acids Res.* **11**, 4049-4063 (1983).
9. Blumer, K. J., Reneke, J. E. & Thorner, J. The STE2 gene product is the ligand-binding component of the alpha-factor receptor of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **263**, 10836-10842 (1988).
10. Hagen, D. C., McCaffrey, G. & Sprague, G. F., Jr. Evidence the yeast STE3 gene encodes a receptor for the peptide pheromone a factor: gene sequence and implications for the structure of the presumed receptor. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 1418-1422 (1986).
11. Burkholder, A. C. & Hartwell, L. H. The yeast α -factor receptor: structural properties deduced from the sequence of the STE2 gene. *Nucleic Acids Res.* **13**, 8463-8475 (1985).
12. Hirsch, J. P. & Cross, F. R. The pheromone receptors inhibit the pheromone response pathway in *Saccharomyces cerevisiae* by a process that is independent of their associated G alpha protein. *Genetics* **135**, 943-953 (1993).
13. Chang, F. & Herskowitz, I. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. *Cell* **63**, 999-1011 (1990).

14. Butty, A. C., Pryciak, P. M., Huang, L. S., Herskowitz, I. & Peter, M. The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. *Science* **282**, 1511-1516 (1998).
15. Segall, J. E. Polarization of yeast cells in spatial gradients of alpha mating factor. *Proceedings of the National Academy of Sciences* **90**, 8332-8336 (1993).
16. Ydenberg, C. A. & Rose, M. D. Yeast mating: a model system for studying cell and nuclear fusion. *Methods Mol. Biol.* **475**, 3-20 (2008).
17. Lesage, G. & Bussey, H. Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **70**, 317-343 (2006).
18. Haber, J. E. Mating-type gene switching in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **32**, 561-599 (1998).
19. Nakafuku, M., Itoh, H., Nakamura, S. & Kaziro, Y. Occurrence in *Saccharomyces cerevisiae* of a gene homologous to the cDNA coding for the alpha subunit of mammalian G proteins. *Proceedings of the National Academy of Sciences* **84**, 2140-2144 (1987).
20. Miyajima, I. *et al.* *GPA1*, a haploid-specific essential gene, encodes a yeast homolog of mammalian G protein which may be involved in mating factor signal transduction. *Cell* **50**, 1011-1019 (1987).
21. Whiteway, M. *et al.* The STE4 and STE18 genes of yeast encode potential β and γ subunits of the mating factor receptor-coupled G protein. *Cell* **56**(3), 467-477 (1989).
22. Nomoto, S., Nakayama, N., Arai, K. & Matsumoto, K. Regulation of the yeast pheromone response pathway by G protein subunits. *EMBO J.* **9**, 691 (1990).
23. Hirschman, J. E. & Jenness, D. D. Dual lipid modification of the yeast $G\gamma$ subunit Ste18p determines membrane localization of $G\beta\gamma$. *Molecular and Cellular Biology* **19**(11), 7705-7711 (1999).
24. Pryciak, P. M. & Huntress, F. A. Membrane recruitment of the kinase cascade scaffold protein Ste5 by the $G\beta\gamma$ complex underlies activation of the yeast pheromone response pathway. *Genes Dev.* **12**, 2684-2697 (1998).
25. Bhattacharjya, S., Gingras, R. & Xu, P. An NMR-based identification of a peptide fragment from the β -subunit of a G-protein showing specific interactions with the GBB domain of the Ste20 kinase in budding yeast. *Biochem. Biophys. Res. Commun.* **347**, 1145-1150 (2006).
26. Leeuw, T. *et al.* Interaction of a G-protein β -subunit with a conserved sequence in Ste20/PAK family protein kinases. *Nature* **391**, 191-195 (1998).

27. van Drogen, F. *et al.* Phosphorylation of the MEKK Ste11p by the PAK-like kinase Ste20p is required for MAP kinase signaling in vivo. *Current Biology* **10**, 630-639 (2000).
28. Choi, K., Satterberg, B., Lyons, D. M. & Elion, E. A. Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisiae*. *Cell* **78**, 499-512 (1994).
29. Marcus, S., Polverino, A., Barr, M. & Wigler, M. Complexes between STE5 and components of the pheromone-responsive mitogen-activated protein kinase module. *Proceedings of the National Academy of Sciences* **91**, 7762-7766 (1994).
30. Printen, J. A. & Sprague, G. Protein-protein interactions in the yeast pheromone response pathway: Ste5p interacts with all members of the MAP kinase cascade. *Genetics* **138**, 609-619 (1994).
31. Rad, M. R., Xu, G. & Hollenberg, C. P. STE50, a novel gene required for activation of conjugation at an early step in mating in *Saccharomyces cerevisiae*. *Molecular and General Genetics MGG* **236**, 145-154 (1992).
32. Wu, C., Leberer, E., Thomas, D. Y. & Whiteway, M. Functional characterization of the interaction of Ste50p with Ste11p MAPKKK in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **10**, 2425-2440 (1999).
33. Xu, G., Jansen, G., Thomas, D. Y., Hollenberg, C. P. & Rad, M. R. Ste50p sustains mating pheromone-induced signal transduction in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **20**, 773-783 (1996).
34. Johnson, D. I. & Pringle, J. R. Molecular characterization of CDC42, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. *J. Cell Biol.* **111**, 143-152 (1990).
35. Lamson, R. E., Winters, M. J. & Pryciak, P. M. Cdc42 regulation of kinase activity and signaling by the yeast p21-activated kinase Ste20. *Mol. Cell. Biol.* **22**, 2939-2951 (2002).
36. Neiman, A. M. & Herskowitz, I. Reconstitution of a yeast protein kinase cascade in vitro: activation of the yeast MEK homologue STE7 by STE11. *Proceedings of the National Academy of Sciences* **91**, 3398-3402 (1994).
37. Cairns, B., Ramer, S. & Kornberg, R. Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of the STE11 kinase and the multiple phosphorylation of the STE7 kinase. *Genes Dev.* **6**, 1305-1318 (1992).
38. Errede, B., Gartner, A., Zhou, Z., Nasmyth, K. & Ammerer, G. MAP kinase-related FUS3 from *S. cerevisiae* is activated by STE7 in vitro. *Nature* **362**, 261-264 (1993).
39. Blackwell, E. *et al.* Effect of the pheromone-responsive Gα and phosphatase proteins of *Saccharomyces cerevisiae* on the subcellular localization of the Fus3 mitogen-activated protein kinase. *Mol. Cell. Biol.* **23**, 1135-1150 (2003).

40. Choi, K., Kranz, J. E., Mahanty, S. K., Park, K. & Elion, E. A. Characterization of Fus3 localization: active Fus3 localizes in complexes of varying size and specific activity. *Mol. Biol. Cell* **10**, 1553-1568 (1999).
41. van Drogen, F., Stucke, V. M., Jorritsma, G. & Peter, M. MAP kinase dynamics in response to pheromones in budding yeast. *Nat. Cell Biol.* **3**, 1051-1059 (2001).
42. Tedford, K., Kim, S., Sa, D., Stevens, K. & Tyers, M. Regulation of the mating pheromone and invasive growth responses in yeast by two MAP kinase substrates. *Current Biology* **7**, 228-238 (1997).
43. Olson, K. A. *et al.* Two regulators of Ste12p inhibit pheromone-responsive transcription by separate mechanisms. *Mol. Cell. Biol.* **20**, 4199-4209 (2000).
44. Elion, E., Satterberg, B. & Kranz, J. FUS3 phosphorylates multiple components of the mating signal transduction cascade: evidence for STE12 and FAR1. *Mol. Biol. Cell* **4**, 495 (1993).
45. Dolan, J. W., Kirkman, C. & Fields, S. The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. *Proceedings of the National Academy of Sciences* **86**, 5703-5707 (1989).
46. Gartner, A. *et al.* Pheromone-dependent G1 cell cycle arrest requires Far1 phosphorylation, but may not involve inhibition of Cdc28-Cln2 kinase, in vivo. *Mol. Cell. Biol.* **18**, 3681-3691 (1998).
47. Peter, M. & Herskowitz, I. Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. *Science* **265**, 1228-1231 (1994).
48. Heinrich, M., Köhler, T. & Mösch, H. Role of Cdc42-Cla4 interaction in the pheromone response of *Saccharomyces cerevisiae*. *Eukaryotic cell* **6**, 317-327 (2007).
49. Tyers, M. & Futcher, B. Far1 and Fus3 link the mating pheromone signal transduction pathway to three G1-phase Cdc28 kinase complexes. *Mol. Cell. Biol.* **13**, 5659-5669 (1993).
50. Jeoung, D., Oehlen, L. & Cross, F. R. Cln3-Associated Kinase Activity in *Saccharomyces cerevisiae* Is Regulated by the Mating Factor Pathway. *Mol. Cell. Biol.* **18**, 433-441 (1998).
51. Blondel, M. *et al.* Nuclear export of Far1p in response to pheromones requires the export receptor Msn5p/Ste21p. *Genes Dev.* **13**, 2284-2300 (1999).
52. Zheng, Y., Cerione, R. & Bender, A. Control of the yeast bud-site assembly GTPase Cdc42. Catalysis of guanine nucleotide exchange by Cdc24 and stimulation of GTPase activity by Bem3. *J. Biol. Chem.* **269**, 2369-2372 (1994).

53. Sloat, B. F. A mutant of yeast defective in cellular morphogenesis. *Science* **200**, 1171-1173 (1978).
54. Shimada, Y., Gulli, M. & Peter, M. Nuclear sequestration of the exchange factor Cdc24 by Far1 regulates cell polarity during yeast mating. *Nat. Cell Biol.* **2**, 117-124 (2000).
55. Nern, A. & Arkowitz, R. A. A Cdc24p-Far1p-G β γ protein complex required for yeast orientation during mating. *J. Cell Biol.* **144**, 1187-1202 (1999).
56. Simon, M. *et al.* Role for the Rho-family GTPase Cdc42 in yeast mating-pheromone signal pathway. *Nature* **376**, 702-705 (1995).
57. Zhao, Z., Leung, T., Manser, E. & Lim, L. Pheromone signalling in *Saccharomyces cerevisiae* requires the small GTP-binding protein Cdc42p and its activator CDC24. *Mol. Cell. Biol.* **15**, 5246-5257 (1995).
58. Nern, A. & Arkowitz, R. A. A GTP-exchange factor required for cell orientation. *Nature* **391**, 195-198 (1998).
59. Cvrckova, F., De Virgilio, C., Manser, E., Pringle, J. R. & Nasmyth, K. Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. *Genes Dev.* **9**, 1817-1830 (1995).
60. Yorihuzi, T. & Ohsumi, Y. *Saccharomyces cerevisiae* mata mutant cells defective in pointed projection formation in response to α -factor at high concentrations. *Yeast* **10**, 579-594 (1994).
61. Evangelista, M. *et al.* Bni1p, a yeast formin linking cdc42p and the actin cytoskeleton during polarized morphogenesis. *Science* **276**, 118-122 (1997).
62. Brown, J. L., Jaquenoud, M., Gulli, M., Chant, J. & Peter, M. Novel Cdc42-binding proteins Gic1 and Gic2 control cell polarity in yeast. *Genes Dev.* **11**, 2972-2982 (1997).
63. Smith, M. G., Swamy, S. R. & Pon, L. A. The life cycle of actin patches in mating yeast. *J. Cell. Sci.* **114**, 1505-1513 (2001).
64. Pruyne, D. W., Schott, D. H. & Bretscher, A. Tropomyosin-containing actin cables direct the Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. *J. Cell Biol.* **143**, 1931-1945 (1998).
65. Chang, F. & Peter, M. Yeasts make their mark. *Nat. Cell Biol.* **5**, 294-299 (2003).
66. Brizzio, V., Gammie, A. E., Nijbroek, G., Michaelis, S. & Rose, M. D. Cell fusion during yeast mating requires high levels of α -factor mating pheromone. *J. Cell Biol.* **135**, 1727-1739 (1996).

67. Adamo, J. E. *et al.* Yeast Cdc42 functions at a late step in exocytosis, specifically during polarized growth of the emerging bud. *J. Cell Biol.* **155**, 581-592 (2001).
68. Zhang, X. *et al.* Cdc42 interacts with the exocyst and regulates polarized secretion. *J. Biol. Chem.* **276**, 46745-46750 (2001).
69. Ayscough, K. R. & Drubin, D. G. A role for the yeast actin cytoskeleton in pheromone receptor clustering and signalling. *Current biology* **8**, 927-931 (1998).
70. Jackson, C. L. & Hartwell, L. H. Courtship in *S. cerevisiae*: both cell types choose mating partners by responding to the strongest pheromone signal. *Cell* **63**, 1039-1051 (1990).
71. Moore, T. I., Chou, C., Nie, Q., Jeon, N. L. & Yi, T. Robust spatial sensing of mating pheromone gradients by yeast cells. *PLoS One* **3**, e3865 (2008).
72. Bagnat, M. & Simons, K. Cell surface polarization during yeast mating. *Proceedings of the National Academy of Sciences* **99**, 14183-14188 (2002).
73. Proszynski, T. J., Klemm, R., Bagnat, M., Gaus, K. & Simons, K. Plasma membrane polarization during mating in yeast cells. *J. Cell Biol.* **173**, 861-866 (2006).
74. Heiman, M. G. & Walter, P. Prm1p, a pheromone-regulated multispanning membrane protein, facilitates plasma membrane fusion during yeast mating. *J. Cell Biol.* **151**, 719-730 (2000).
75. Chan, R. K. & Otte, C. A. Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by a factor and alpha factor pheromones. *Mol. Cell. Biol.* **2**, 11-20 (1982).
76. Chan, R. K. & Otte, C. A. Physiological characterization of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by a factor and alpha factor pheromones. *Mol. Cell. Biol.* **2**, 21 (1982).
77. Apanovitch, D. M., Slep, K. C., Sigler, P. B. & Dohlman, H. G. Sst2 is a GTPase-activating protein for Gpa1: purification and characterization of a cognate RGS-G α protein pair in yeast. *Biochemistry (N. Y.)* **37**, 4815-4822 (1998).
78. Dohlman, H. G., Song, J., Ma, D., Courchesne, W. E. & Thorner, J. Sst2, a negative regulator of pheromone signaling in the yeast *Saccharomyces cerevisiae*: expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein alpha subunit). *Mol. Cell. Biol.* **16**, 5194-5209 (1996).
79. Siekhaus, D. E. & Drubin, D. G. Spontaneous receptor-independent heterotrimeric G-protein signalling in an RGS mutant. *Nat. Cell Biol.* **5**, 231-235 (2003).

80. Metodiev, M. V., Matheos, D., Rose, M. D. & Stone, D. E. Regulation of MAPK function by direct interaction with the mating-specific $G\alpha$ in yeast. *Science* **296**, 1483-1486 (2002).
81. Sprague Jr, G. F. & Herskowitz, I. Control of yeast cell type by the mating type locus: I. Identification and control of expression of the a -specific gene $BAR1$. *J. Mol. Biol.* **153**, 305-321 (1981).
82. Ciejek, E. & Thorner, J. Recovery of *S. cerevisiae* a cells from G1 arrest by α factor pheromone requires endopeptidase action. *Cell* **18**, 623-635 (1979).
83. Doi, S., Tanabe, K., Watanabe, M., Yamaguchi, M. & Yoshimura, M. An α -specific gene, $SAG1$ is required for sexual agglutination in *Saccharomyces cerevisiae*. *Curr. Genet.* **15**, 393-398 (1989).
84. Lipke, P., Wojciechowicz, D. & Kurjan, J. $AG\alpha 1$ is the structural gene for the *Saccharomyces cerevisiae* α -agglutinin, a cell surface glycoprotein involved in cell-cell interactions during mating. *Mol. Cell. Biol.* **9**, 3155-3165 (1989).
85. Roy, A., Lu, C., Marykwas, D., Lipke, P. & Kurjan, J. The $AGA1$ product is involved in cell surface attachment of the *Saccharomyces cerevisiae* cell adhesion glycoprotein a -agglutinin. *Mol. Cell. Biol.* **11**, 4196-4206 (1991).
86. Cappellaro, C. *et al.* *Saccharomyces cerevisiae* a - and α -agglutinin: characterization of their molecular interaction. *EMBO J.* **10**, 4081 (1991).
87. Erdman, S., Lin, L., Malczynski, M. & Snyder, M. Pheromone-regulated genes required for yeast mating differentiation. *J. Cell Biol.* **140**, 461-483 (1998).
88. Wojciechowicz, D., Lu, C., Kurjan, J. & Lipke, P. Cell surface anchorage and ligand-binding domains of the *Saccharomyces cerevisiae* cell adhesion protein α -agglutinin, a member of the immunoglobulin superfamily. *Mol. Cell. Biol.* **13**, 2554-2563 (1993).
89. Watzele, M., Klis, F. & Tanner, W. Purification and characterization of the inducible a agglutinin of *Saccharomyces cerevisiae*. *EMBO J.* **7**, 1483 (1988).
90. Zhao, H., Shen, Z., Kahn, P. C. & Lipke, P. N. Interaction of α -agglutinin and a -agglutinin, *Saccharomyces cerevisiae* sexual cell adhesion molecules. *J. Bacteriol.* **183**, 2874-2880 (2001).
91. Orlean, P., Ammer, H., Watzele, M. & Tanner, W. Synthesis of an O-glycosylated cell surface protein induced in yeast by α factor. *Proceedings of the National Academy of Sciences* **83**, 6263-6266 (1986).
92. de Nobel, H., Pike, J., Lipke, P. N. & Kurjan, J. Genetics of a -agglutinin function in *Saccharomyces cerevisiae*. *Molecular and General Genetics MGG* **247**, 409-415 (1995).

93. Guo, B., Styles, C. A., Feng, Q. & Fink, G. R. A *Saccharomyces* gene family involved in invasive growth, cell–cell adhesion, and mating. *Proceedings of the National Academy of Sciences* **97**, 12158-12163 (2000).
94. Zhang, M., Bennett, D. & Erdman, S. E. Maintenance of mating cell integrity requires the adhesin Fig2p. *Eukaryotic cell* **1**, 811-822 (2002).
95. Dupres, V., Dufrêne, Y. F. & Heinisch, J. J. Measuring cell wall thickness in living yeast cells using single molecular rulers. *ACS nano* **4**, 5498-5504 (2010).
96. Philips, J. & Herskowitz, I. Osmotic balance regulates cell fusion during mating in *Saccharomyces cerevisiae*. *J. Cell Biol.* **138**, 961-974 (1997).
97. de Nobel, H. *et al.* Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in FKS2-lacZ expression, glucanase resistance and thermotolerance. *Microbiology* **146** (Pt 9), 2121-2132 (2000).
98. Trueheart, J., Boeke, J. D. & Fink, G. R. Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. *Mol. Cell. Biol.* **7**, 2316-2328 (1987).
99. Trueheart, J. & Fink, G. R. The yeast cell fusion protein FUS1 is O-glycosylated and spans the plasma membrane. *Proceedings of the National Academy of Sciences* **86**, 9916-9920 (1989).
100. Santos, B. & Snyder, M. Specific protein targeting during cell differentiation: polarized localization of Fus1p during mating depends on Chs5p in *Saccharomyces cerevisiae*. *Eukaryotic cell* **2**, 821-825 (2003).
101. Trautwein, M. *et al.* Arf1p, Chs5p and the ChAPs are required for export of specialized cargo from the Golgi. *EMBO J.* **25**, 943-954 (2006).
102. Nelson, B. *et al.* Fus1p interacts with components of the Hog1p mitogen-activated protein kinase and Cdc42p morphogenesis signaling pathways to control cell fusion during yeast mating. *Genetics* **166**, 67-77 (2004).
103. Gammie, A. E., Brizzio, V. & Rose, M. D. Distinct morphological phenotypes of cell fusion mutants. *Mol. Biol. Cell* **9**, 1395-1410 (1998).
104. Maeda, T., Takekawa, M. & Saito, H. Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. *Science* **269**, 554-558 (1995).
105. Sheu, Y., Barral, Y. & Snyder, M. Polarized growth controls cell shape and bipolar bud site selection in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **20**, 5235-5247 (2000).
106. Dorer, R., Boone, C., Kimbrough, T., Kim, J. & Hartwell, L. H. Genetic analysis of default mating behavior in *Saccharomyces cerevisiae*. *Genetics* **146**, 39-55 (1997).

107. Kim, J. & Rose, M. D. A mechanism for the coordination of proliferation and differentiation by spatial regulation of Fus2p in budding yeast. *Genes Dev.* **26**, 1110-1121 (2012).
108. Ydenberg, C. A. & Rose, M. D. Antagonistic regulation of Fus2p nuclear localization by pheromone signaling and the cell cycle. *J. Cell Biol.* **184**, 409-422 (2009).
109. Brizzio, V., Gammie, A. E. & Rose, M. D. Rvs161p interacts with Fus2p to promote cell fusion in *Saccharomyces cerevisiae*. *J. Cell Biol.* **141**, 567-584 (1998).
110. Bon, E. *et al.* A network of proteins around Rvs167p and Rvs161p, two proteins related to the yeast actin cytoskeleton. *Yeast* **16**, 1229-1241 (2000).
111. Friesen, H. *et al.* Characterization of the yeast amphiphysins Rvs161p and Rvs167p reveals roles for the Rvs heterodimer in vivo. *Mol. Biol. Cell* **17**, 1306-1321 (2006).
112. Peter, B. J. *et al.* BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. *Science* **303**, 495-499 (2004).
113. Crouzet, M., Urdaci, M., Dulau, L. & Aigle, M. Yeast mutant affected for viability upon nutrient starvation: characterization and cloning of the RVS161 gene. *Yeast* **7**, 727-743 (1991).
114. Kurihara, L. J., Beh, C. T., Latterich, M., Schekman, R. & Rose, M. D. Nuclear congression and membrane fusion: two distinct events in the yeast karyogamy pathway. *J. Cell Biol.* **126**, 911 (1994).
115. Sheltzer, J. M. & Rose, M. D. The class V myosin Myo2p is required for Fus2p transport and actin polarization during the yeast mating response. *Mol. Biol. Cell* **20**, 2909-2919 (2009).
116. Paterson, J. M., Ydenberg, C. A. & Rose, M. D. Dynamic localization of yeast Fus2p to an expanding ring at the cell fusion junction during mating. *J. Cell Biol.* **181**, 697-709 (2008).
117. Barale, S., McCusker, D. & Arkowitz, R. A. The exchange factor Cdc24 is required for cell fusion during yeast mating. *Eukaryotic cell* **3**, 1049-1061 (2004).
118. Barale, S., McCusker, D. & Arkowitz, R. A. Cdc42p GDP/GTP cycling is necessary for efficient cell fusion during yeast mating. *Mol. Biol. Cell* **17**, 2824-2838 (2006).
119. Ydenberg, C. A., Stein, R. A. & Rose, M. D. Cdc42p and Fus2p act together late in yeast cell fusion. *Mol. Biol. Cell* **23**, 1208-1218 (2012).
120. Fitch, P. G., Gammie, A. E., Lee, D. J., de Candal, V. B. & Rose, M. D. Lrg1p Is a Rho1 GTPase-activating protein required for efficient cell fusion in yeast. *Genetics* **168**, 733-746 (2004).

121. Cappellaro, C., Mersa, V. & Tanner, W. New potential cell wall glucanases of *Saccharomyces cerevisiae* and their involvement in mating. *J. Bacteriol.* **180**, 5030-5037 (1998).
122. Zeitlinger, J. *et al.* Program-specific distribution of a transcription factor dependent on partner transcription factor and MAPK signaling. *Cell* **113**, 395-404 (2003).
123. Lee, J. & Lentz, B. R. Evolution of lipidic structures during model membrane fusion and the relation of this process to cell membrane fusion. *Biochemistry (N. Y.)* **36**, 6251-6259 (1997).
124. Chernomordik, L. V. & Kozlov, M. M. Mechanics of membrane fusion. *Nature structural & molecular biology* **15**, 675-683 (2008).
125. Engel, A. & Walter, P. Membrane lysis during biological membrane fusion: collateral damage by misregulated fusion machines. *J. Cell Biol.* **183**, 181-186 (2008).
126. Olmo, V. N. & Grote, E. Prm1 functions as a disulfide-linked complex in yeast mating. *J. Biol. Chem.* **285**, 2274-2283 (2010).
127. Muller, E. M., Mackin, N. A., Erdman, S. E. & Cunningham, K. W. Fig1p facilitates Ca²⁺ influx and cell fusion during mating of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **278**, 38461-38469 (2003).
128. Aguilar, P. S., Engel, A. & Walter, P. The plasma membrane proteins Prm1 and Fig1 ascertain fidelity of membrane fusion during yeast mating. *Mol. Biol. Cell* **18**, 547-556 (2007).
129. Jin, H., Carlile, C., Nolan, S. & Grote, E. Prm1 prevents contact-dependent lysis of yeast mating pairs. *Eukaryotic cell* **3**, 1664-1673 (2004).
130. Steinhardt, R. A., Bi, G. & Alderton, J. M. Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release. *Science* **263**, 390-393 (1994).
131. Yawo, H. & Kuno, M. Calcium dependence of membrane sealing at the cut end of the cockroach giant axon. *The Journal of neuroscience* **5**, 1626-1632 (1985).
132. Engel, A., Aguilar, P. S. & Walter, P. The yeast cell fusion protein Prm1p requires covalent dimerization to promote membrane fusion. *PLoS one* **5**, e10593 (2010).
133. Heiman, M. G., Engel, A. & Walter, P. The Golgi-resident protease Kex2 acts in conjunction with Prm1 to facilitate cell fusion during yeast mating. *J. Cell Biol.* **176**, 209-222 (2007).
134. Wilcox, C. A. & Fuller, R. S. Posttranslational processing of the prohormone-cleaving Kex2 protease in the *Saccharomyces cerevisiae* secretory pathway. *J. Cell Biol.* **115**, 297-307 (1991).
135. Fuller, R. S., Sterne, R. E. & Thorner, J. Enzymes required for yeast prohormone processing. *Annu. Rev. Physiol.* **50**, 345-362 (1988).

136. Fuller, R. S., Brake, A. & Thorner, J. Yeast prohormone processing enzyme (KEX2 gene product) is a Ca²⁺-dependent serine protease. *Proceedings of the National Academy of Sciences* **86**, 1434-1438 (1989).
137. Fuller, R. S., Brake, A. J. & Thorner, J. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science* **246**, 482-486 (1989).
138. Julius, D., Brake, A., Blair, L., Kunisawa, R. & Thorner, J. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro- α -factor. *Cell* **37**, 1075-1089 (1984).
139. Jin, H., McCaffery, J. M. & Grote, E. Ergosterol promotes pheromone signaling and plasma membrane fusion in mating yeast. *J. Cell Biol.* **180**, 813-826 (2008).
140. Nolan, S., Cowan, A. E., Koppel, D. E., Jin, H. & Grote, E. FUS1 regulates the opening and expansion of fusion pores between mating yeast. *Mol. Biol. Cell* **17**, 2439-2450 (2006).
141. Lahav, R., Gammie, A., Tavazoie, S. & Rose, M. D. Role of transcription factor Kar4 in regulating downstream events in the *Saccharomyces cerevisiae* pheromone response pathway. *Mol. Cell. Biol.* **27**, 818-829 (2007).
142. Molk, J. N. & Bloom, K. Microtubule dynamics in the budding yeast mating pathway. *J. Cell. Sci.* **119**, 3485-3490 (2006).
143. Schwartz, K., Richards, K. & Botstein, D. BIM1 encodes a microtubule-binding protein in yeast. *Mol. Biol. Cell* **8**, 2677-2691 (1997).
144. Tirnauer, J. S., O'Toole, E., Berrueta, L., Bierer, B. E. & Pellman, D. Yeast Bim1p promotes the G1-specific dynamics of microtubules. *J. Cell Biol.* **145**, 993-1007 (1999).
145. Korinek, W. S., Copeland, M. J., Chaudhuri, A. & Chant, J. Molecular linkage underlying microtubule orientation toward cortical sites in yeast. *Science* **287**, 2257-2259 (2000).
146. Miller, R. K., Cheng, S. & Rose, M. D. Bim1p/Yeb1p mediates the Kar9p-dependent cortical attachment of cytoplasmic microtubules. *Mol. Biol. Cell* **11**, 2949-2959 (2000).
147. Hwang, E., Kusch, J., Barral, Y. & Huffaker, T. C. Spindle orientation in *Saccharomyces cerevisiae* depends on the transport of microtubule ends along polarized actin cables. *J. Cell Biol.* **161**, 483-488 (2003).
148. Yin, H., Pruyne, D., Huffaker, T. C. & Bretscher, A. Myosin V orientates the mitotic spindle in yeast. *Nature* **406**, 1013-1015 (2000).
149. Miller, R. K., Matheos, D. & Rose, M. D. The cortical localization of the microtubule orientation protein, Kar9p, is dependent upon actin and proteins required for polarization. *J. Cell Biol.* **144**, 963-975 (1999).

150. Page, B. D., Satterwhite, L. L., Rose, M. D. & Snyder, M. Localization of the Kar3 kinesin heavy chain-related protein requires the Cik1 interacting protein. *J. Cell Biol.* **124**, 507-519 (1994).
151. Sproul, L. R., Anderson, D. J., Mackey, A. T., Saunders, W. S. & Gilbert, S. P. Cik1 targets the minus-end kinesin depolymerase kar3 to microtubule plus ends. *Current biology* **15**, 1420-1427 (2005).
152. Maddox, P. S., Stemple, J. K., Satterwhite, L., Salmon, E. D. & Bloom, K. The minus end-directed motor Kar3 is required for coupling dynamic microtubule plus ends to the cortical shmoo tip in budding yeast. *Current Biology* **13**, 1423-1428 (2003).
153. Zaichick, S. V. *et al.* The mating-specific G α interacts with a kinesin-14 and regulates pheromone-induced nuclear migration in budding yeast. *Mol. Biol. Cell* **20**, 2820-2830 (2009).
154. Molk, J. N., Salmon, E. & Bloom, K. Nuclear congression is driven by cytoplasmic microtubule plus end interactions in *S. cerevisiae*. *J. Cell Biol.* **172**, 27-39 (2006).
155. Gibeaux, R., Politi, A. Z., Nédélec, F., Antony, C. & Knop, M. Spindle pole body-anchored Kar3 drives the nucleus along microtubules from another nucleus in preparation for nuclear fusion during yeast karyogamy. *Genes Dev.* **27**, 335-349 (2013).
156. Melloy, P., Shen, S., White, E., McIntosh, J. R. & Rose, M. D. Nuclear fusion during yeast mating occurs by a three-step pathway. *J. Cell Biol.* **179**, 659-670 (2007).
157. Melloy, P., Shen, S., White, E. & Rose, M. D. Distinct roles for key karyogamy proteins during yeast nuclear fusion. *Mol. Biol. Cell* **20**, 3773-3782 (2009).
158. Shen, S., Tobery, C. E. & Rose, M. D. Prm3p is a pheromone-induced peripheral nuclear envelope protein required for yeast nuclear fusion. *Mol. Biol. Cell* **20**, 2438-2450 (2009).
159. Nishikawa, S., Hirata, A. & Endo, T. Nuclear inner membrane fusion facilitated by yeast Jem1p is required for spindle pole body fusion but not for the first mitotic nuclear division during yeast mating. *Genes to Cells* **13**, 1185-1195 (2008).
160. Nishikawa, S. & Endo, T. The yeast JEM1p is a DnaJ-like protein of the endoplasmic reticulum membrane required for nuclear fusion. *J. Biol. Chem.* **272**, 12889-12892 (1997).
161. Tokunaga, M., Kawamura, A. & Kohno, K. Purification and characterization of BiP/Kar2 protein from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **267**, 17553-17559 (1992).
162. Brizzio, V. *et al.* Genetic interactions between KAR7/SEC71, KAR8/JEM1, KAR5, and KAR2 during nuclear fusion in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **10**, 609-626 (1999).
163. Nishikawa, S. *et al.* Nep98p is a component of the yeast spindle pole body and essential for nuclear division and fusion. *J. Biol. Chem.* **278**, 9938-9943 (2003).

164. Friederichs, J. M. *et al.* The SUN protein Mps3 is required for spindle pole body insertion into the nuclear membrane and nuclear envelope homeostasis. *PLoS genetics* **7**, e1002365 (2011).
165. Kim, J. *et al.* Localization and Signaling of Gbeta Subunit Ste4p Are Controlled by a-Factor Receptor and the a-Specific Protein Asg7p. *Science Signaling* **20**, 8826 (2000).
166. Roth, A. F., Nelson, B., Boone, C. & Davis, N. G. Asg7p-Ste3p inhibition of pheromone signaling: regulation of the zygotic transition to vegetative growth. *Science Signaling* **20**, 8815 (2000).
167. Galgoczy, D. J. *et al.* Genomic dissection of the cell-type-specification circuit in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences* **101**, 18069-18074 (2004).
168. Strathern, J., Hicks, J. & Herskowitz, I. Control of cell type in yeast by the mating type locus: the $\alpha 1$ - $\alpha 2$ hypothesis. *J. Mol. Biol.* **147**, 357-372 (1981).
169. Tatchell, K., Nasmyth, K. A., Hall, B. D., Astell, C. & Smith, M. In vitro mutation analysis of the mating-type locus in yeast. *Cell* **27**, 25-35 (1981).
170. Herskowitz, I. & Oshima, Y. Control of cell type in *Saccharomyces cerevisiae*: mating type and mating-type interconversion. *Cold Spring Harbor Monograph Archive* **11**, 181-209 (1981).
171. Goutte, C. & Johnson, A. D. $\alpha 1$ protein alters the DNA binding specificity of $\alpha 2$ repressor. *Cell* **52**, 875-882 (1988).
172. Roberts, C. J. *et al.* Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles. *Science Signaling* **287**, 873 (2000).
173. Michaelis, S. & Herskowitz, I. The a-factor pheromone of *Saccharomyces cerevisiae* is essential for mating. *Mol. Cell. Biol.* **8**, 1309-1318 (1988).
174. McGrath, J. P. & Varshavsky, A. The yeast STE6 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. *Nature* **340**, 400-404 (1989).
175. Bender, A. & Sprague, G. Pheromones and pheromone receptors are the primary determinants of mating specificity in the yeast *Saccharomyces cerevisiae*. *Genetics* **121**, 463-476 (1989).
176. MacKay, V. L. *et al.* The *Saccharomyces cerevisiae* BAR1 gene encodes an exported protein with homology to pepsin. *Proceedings of the National Academy of Sciences* **85**, 55-59 (1988).
177. Barkai, N., Rose, M. D. & Wingreen, N. S. Protease helps yeast find mating partners. *Nature* **396**, 422-423 (1998).

178. Rappaport, N. & Barkai, N. Disentangling signaling gradients generated by equivalent sources. *J. Biol. Phys.* **38**, 267-278 (2012).
179. Jin, M. *et al.* Yeast dynamically modify their environment to achieve better mating efficiency. *Science signaling* **4**, ra54 (2011).
180. Zhong, H., McCord, R. & Vershon, A. K. Identification of target sites of the $\alpha 2$ -Mcm1 repressor complex in the yeast genome. *Genome Res.* **9**, 1040-1047 (1999).
181. Kurjan, J. Alpha-factor structural gene mutations in *Saccharomyces cerevisiae*: effects on alpha-factor production and mating. *Mol. Cell. Biol.* **5**, 787-796 (1985).
182. Marcus, S., Xue, C., Naider, F. & Becker, J. Degradation of a-factor by a *Saccharomyces cerevisiae* alpha-mating-type-specific endopeptidase: evidence for a role in recovery of cells from G1 arrest. *Mol. Cell. Biol.* **11**, 1030-1039 (1991).
183. Steden, M., Betz, R. & Duntze, W. Isolation and characterization of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by the mating hormone a-factor. *Molecular and General Genetics MGG* **219**, 439-444 (1989).
184. Brar, G. A. *et al.* High-resolution view of the yeast meiotic program revealed by ribosome profiling. *Science* **335**, 552-557 (2012).
185. Giaever, G. *et al.* Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**, 387-391 (2002).
186. Hamada, K., Terashima, H., Arisawa, M., Yabuki, N. & Kitada, K. Amino acid residues in the ω -minus region participate in cellular localization of yeast glycosylphosphatidylinositol-attached proteins. *J. Bacteriol.* **181**, 3886-3889 (1999).

CHAPTER 2

Genetically engineered transvestites reveal novel mating genes in budding yeast

ABSTRACT

Mating type in yeast is controlled by transcription factors expressed from the *MAT* locus; however, the characterization of the genes these factors regulate necessary to define a mating type is incomplete. Each mating type expresses a pheromone which binds to a G-protein coupled receptor expressed by the opposite mating type, and signaling through these receptors is essential for efficient mating. To find additional mating type-specific proteins required for efficient zygote formation, we have constructed transvestite strains that swap the pheromone, pheromone receptor, and pheromone processing factors of one mating type for another. Using these strains we were able to compare gene expression in pheromone-stimulated *MAT α* and *MAT \mathbf{a}* cells and identified a novel *MAT α* -specific protein that interferes with **a**-factor, which we have named *AFBI*. Although the transvestite strains were capable of mating, the mating efficiency of these cells was low. Through characterization of the transvestite cells, we provide further evidence that all of the *MAT \mathbf{a}* -specific proteins have been identified and that sufficient α -factor expression is necessary for efficient mating. We also show that while changing the regulation of various genes involved with pheromone induction does not cause sterility, it can cause a significant decrease in mating efficiency, arguing that mating is a fragile process.

INTRODUCTION

Many higher eukaryotes reproduce when gametes produced by individuals from two different genders come in contact and fuse. However, genetic studies of this process are difficult because sterile mutants cannot reproduce. The budding yeast, *Saccharomyces cerevisiae*, is an attractive solution to this problem because although it is able to replicate sexually when two haploid cells of opposite mating types (*MATa* and *MAT α*) come in contact with one another and fuse to form a single diploid cell, it can replicate asexually in both its diploid and haploid states¹.² Mating type is controlled by transcription factors expressed from the mating type (*MAT*) locus³; however, we do not know if the list of genes that these factors control to define a mating type is complete.

Mating depends on pheromone-mediated, intercellular communication. Each mating type expresses a pheromone which binds to a G-protein coupled receptor expressed by the opposite mating type, and signaling through these receptors is essential for mating⁴. *MAT α* cells produce α -factor^{5,6} and express the **a**-factor receptor, Ste3, on their surface⁷ (Figure 2.1A). *MATa* cells produce **a**-factor⁸ and express the α -factor receptor, Ste2, on their surface^{9,10} (Figure 2.1A). In the absence of *MAT α* cells, *MATa* cells express **a**-factor and Ste2 at low levels^{11,12}, but when Ste2 binds α -factor, a mitogen-activated protein (MAP) kinase cascade, which is conserved in other ascomycete fungi, is started, stimulating the cell to produce higher levels of its own pheromone, **a**-factor, and other proteins involved with mating^{4,13-15}. Pheromone stimulation arrests *MATa* cells in G1¹⁶, polarizes them in the direction of highest pheromone concentration¹⁷, and induces polarized cell growth to form a mating projection known as a shmoo¹⁸. *MAT α* cells produce a similar, coordinated response using the reciprocal G-protein coupled receptor (Ste3) to detect **a**-factor (Figure 2.1A)^{4-7,12,13,17,19}.

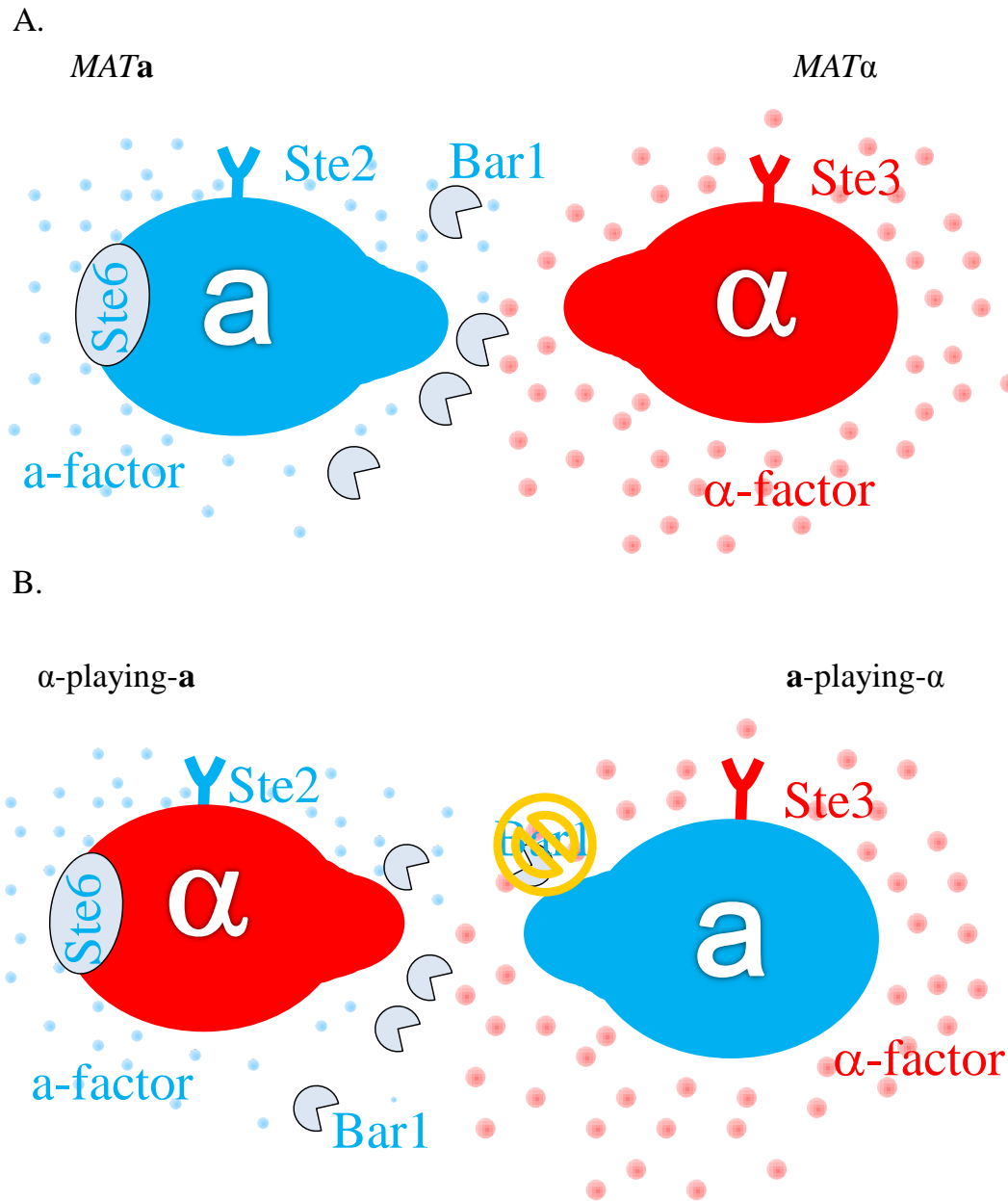


Figure 2.1. Wild-type mating types and transvestite mating types. **A.** *MATa* cells express the pheromone **a**-factor, which is secreted through the ABC transporter, Ste6, the α -factor receptor, Ste2, and the α -factor protease, Bar1. *MAT α* cells express the pheromone α -factor and the **a**-factor receptor, Ste3. **B.** α -playing-**a** cells are *MAT α* cells that express **a**-factor instead of α -factor, Ste2 instead of Ste3, the **a**-factor transporter, Ste6, and the α -factor protease, Bar1. **a**-playing- α cells are *MATa* cells that express α -factor instead of **a**-factor, Ste3 instead of Ste2, and are *bar1 Δ* .

Cells that have mutations in the proteins required for secreting, processing, and responding to pheromone are sterile⁴, and previous work demonstrated that reciprocal pheromones and receptors are the only proteins that are absolutely necessary to form zygotes²⁰. However, there are other mating type-specific proteins, such as the α -factor protease, Bar1^{ref. 21, 22}, and the mating agglutinins²³⁻²⁵ that serve to make mating more efficient. We were interested in identifying additional mating type-specific genes necessary for efficient mating and asking how much the quantitative details of mating gene expression contributes to the efficiency of mating.

To do this, we constructed “transvestite” strains (Figure 2.1B). These genetically engineered strains have one allele at the mating type locus but express the pheromone, pheromone receptor, and proteins responsible for secreting or processing pheromones that are normally induced by the other *MAT* allele; thus, a *MAT* α transvestite would express **a**-factor instead of α -factor and the α -factor receptor (Ste2) instead of the **a**-factor receptor (Ste3). By studying the behavior of these cells, we were able to learn more about the differences between *MAT***a** and *MAT* α cells. *MAT***a** cells that express α -factor and Ste3, which we call **a**-playing- α cells, mate 3 times worse than genuine *MAT* α cells, while *MAT* α cells that express **a**-factor and Ste2, which we call α -playing-**a** cells, mate 50 times worse than genuine *MAT***a** cells. The mating defects of the α -playing-**a** cells are due in part to the expression of a novel, *MAT* α -specific **a**-factor barrier protein. We also show that although α -playing-**a** cells are able to form shmoo in response to α -factor, they only transiently arrest the cell cycle. Additionally, our results show that the quantitative details of pheromone secretion and processing have strong effects on mating efficiency.

RESULTS

Transvestite cells can mate

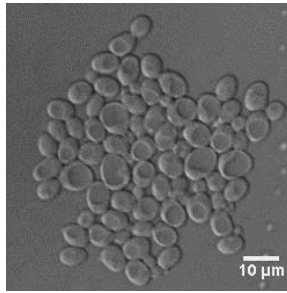
To study mating-type specific genes necessary for efficient mating, we constructed two types of transvestite strains. The first is **a**-playing- α : *MATa* cells that have been engineered to produce α -factor and the **a**-factor receptor by replacing the open reading frames of *STE2* with *STE3*, *MFA1* with *MF α 1*, and *MFA2* with *MF α 2*. We also deleted the α -factor protease, *BARI*, and the gene *ASG7*, which causes internalization of Ste3 and Ste4^{ref. 26}, inhibiting signaling in newly formed zygotes²⁷. However, these cells are still *MATa* at the *MAT* locus (Figure 2.1B). The second type of transvestite is α -playing-**a**. These cells are *MAT α* at the *MAT* locus, but have been engineered to produce **a**-factor and the α -factor receptor by replacing the open reading frame of *STE3* with *STE2*, *MF α 1* with *MFA1*, and *MF α 2* with *MFA2*. We also drove the expression of *BARI* with an engineered version of the haploid specific promoter, *P_{FUS1}*^{ref. 28}, and the expression of the **a**-factor transporter, *STE6* with the *MF α 1* promoter (Figure 2.1B).

We determined whether the manipulated genes are the only mating-type specific proteins required for efficient mating, by crossing these cells with wild-type cells of their original mating type (e.g. α -playing-**a** crossed to *MAT α*). When two wild-type cells mate, although zygotes will initially express the pheromones and receptors of both mating types, the presence of the transcription factors expressed from both the *MATa* and the *MAT α* loci turns off transcription of these genes³, and the zygotes are able to escape pheromone-induced G1 arrest²⁷. Conversely, the zygotes produced by crossing the transvestite cells with cells of the same original mating type will express pheromones and receptors from both mating types but the transcription factors from a single *MAT* locus, raising the concern that these zygotes may be self-stimulating and persist in pheromone-induced G1 arrest. To confirm that diploids formed by mating transvestite cells with wild-type cells are able to divide, we mated the transvestites to wild-type strains with different nutritional auxotrophies and harvested diploids from media lacking both nutrients. We identified

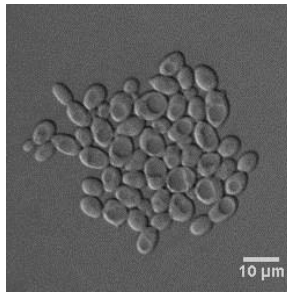
both **a**-playing- α /*MATa* and α -playing-**a**/*MAT α* diploids, which are capable of normal progression through the cell cycle and generally have normal cell morphology (Figure 2.2A and 2.2B). It is possible to find an occasional population of α -playing-**a**/*MAT α* diploids with abnormal morphology, but even these are capable of budding (Figure 2.2C). The ability of these diploids to bud indicates that it is possible to measure the mating efficiency of transvestites crossed with wild-type cells of the same original mating type by complementation of nutritional auxotrophies.

We used quantitative mating assays to measure the mating efficiency of the transvestite cells. Cells of the two mating types are incubated together at high density and then plated on media that distinguishes diploid cells from either parental haploid. When wild-type *MATa* cells are mated with wild-type *MAT α* cells, 63% of haploids form diploids (Figure 2.3). However, the mating efficiency of the **a**-playing- α cells crossed with *MATa* cells is one third that of a wild-type cross, and the mating efficiency of the α -playing-**a** cells crossed with *MAT α* cells is 50-fold lower than the efficiency of a wild-type cross ($p < 10^{-6}$) (Figure 2.3). We also crossed the transvestite strains to each other. Since the diploids formed by this cross will express the transcription factors from both the *MATa* and *MAT α* loci, any diploids that form should not arrest due to self-stimulation, but the efficiency of this cross is more than 500-fold lower than a wild-type cross ($p < 10^{-6}$) (Figure 2.3). Corroborating previous results²⁰, our observation that the transvestite strains are capable of forming viable diploids with cells of the same original mating type shows that there are no additional mating type-specific genes required for zygote formation other than the ones we manipulated. However, the low mating efficiency of the transvestites crossed to wild-type partners and to each other implies that there are additional requirements for efficient mating.

A.



B.



C.

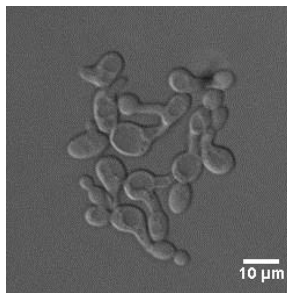


Figure 2.2. Transvestite/wild-type diploids are able to proceed through the cell cycle. **A.** *a*-playing-*α*/*MATα* diploids budding. Cells were grown in YPD and pictures were taken using DIC with 60x magnification. The scale bar indicates 10μm. **B.** *α*-playing-*a*/*MATα* diploids budding. Cells were grown in YPD and pictures were taken using DIC with 60x magnification. The scale bar indicates 10μm. **C.** *α*-playing-*a*/*MATα* diploids budding with abnormal morphology. Cells were grown in YPD and pictures were taken using DIC with 60x magnification. The scale bar indicates 10μm.

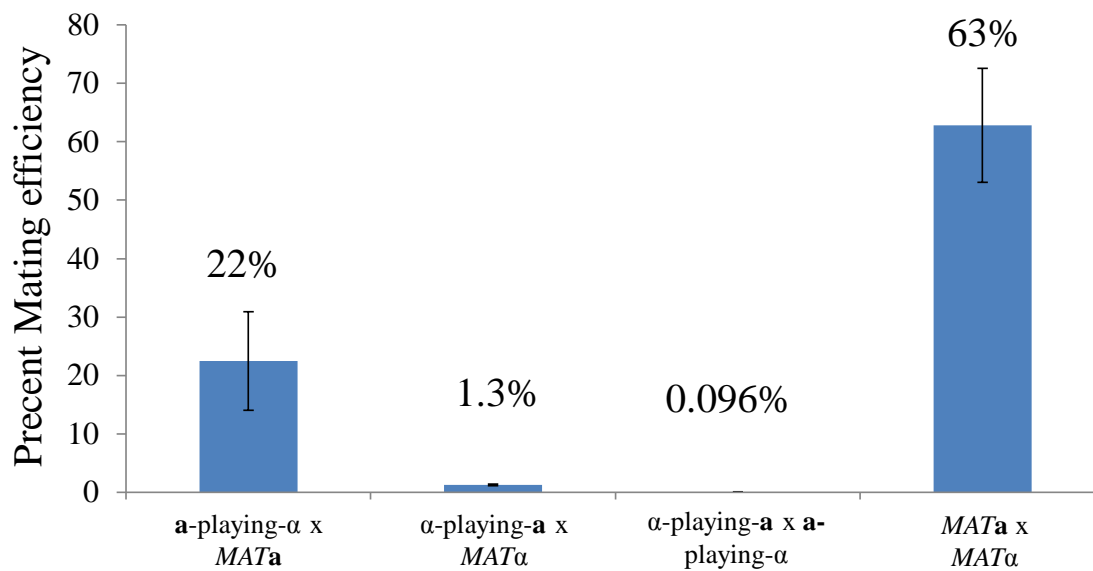


Figure 2.3. Transvestite cells can mate. Mating efficiency of cells with different nutritional auxotrophies grown to log phase and then mixed at a 1:1 ratio on a nitrocellulose filter, which was placed on a YPD plate for 5 hours. Mating efficiencies are the percentage of diploids that form colonies on double dropout plates compared to the number of colonies formed on single dropout plates. Error bars are standard deviations.

***a*-playing- α cells produce too little α -factor**

To further investigate the requirements for efficient mating, we more carefully probed the mating defects of the transvestite cells. *a*-playing- α cells mate 2.8-fold less efficiently than wildtype (Figure 2.3). Because the genes that had been replaced in this strain were those encoding the pheromone and pheromone receptor, the obvious candidates for this difference were the ability of the *a*-playing- α cells to respond to *a*-factor and to produce α -factor.

We began by testing the response to *a*-factor. We made mating mixtures of *a*-playing- α cells expressing YFP under the pheromone-inducible promoter, P_{FUS1} , and *MATa* cells expressing mCherry under the *ACT1* promoter and assayed for the expression of YFP in the *a*-playing- α cells after 2.5 hours. The expression of YFP in the *a*-playing- α cells indicates that the *a*-playing- α cells are able to signal through Ste3 for expression of pheromone-induced genes (Figure 2.4). It was also possible to see the formation of auto-stimulated zygotes, which express both YFP under the *FUS1* promoter and mCherry under the *ACT1* promoter (Figure 2.4). Since the G-protein and downstream components of the pheromone response pathway should be the same in *MATa* and *MAT α* cells⁴, and the cells are able to express and signal through Ste3, we hypothesize that the response of the *a*-playing- α cells to *a*-factor is not causing a mating defect.

Pheromone production is important for zygote formation^{29,30}, and it has been shown that *MATa* cells prefer to mate with the *MAT α* cell that produces the highest amount of α -factor²². Since manipulation of the pheromone genes was integral to the design of the transvestite cells, we next measured the pheromone production of the *a*-playing- α cells. We grew cells in rich media and collected the supernatant, which we then used to stimulate *MATa* cells that are missing the α -factor protease, Bar1. By measuring the fraction of the *MATa* *bar1 Δ* cells that arrest their cell cycles and shmoo (the shmooing index) when exposed to the supernatants of

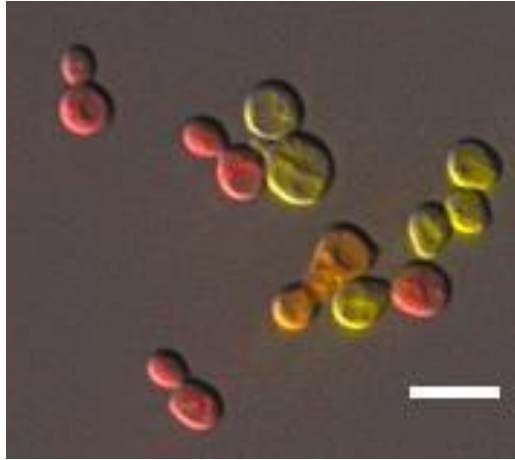


Figure 2.4. a-playing- α cells respond to a-factor. a-playing- α P_{FUS1} -YFP cells in a mating mixture with $MATa$ P_{ACT1} -mCherry cells. Yellow indicates YFP expression. Red indicates mCherry expression. The orange cell is a diploid expressing both YFP and mCherry. The picture was taken 2.5 hours after mixing the cells using DIC and fluorescence at 20x magnification. The scale bar indicates 10 μ m.

various strains and comparing this data to a standard curve generated with known quantities of synthetic α -factor, we are able to measure the amount of α -factor produced by these cells.

Unstimulated, **a**-playing- α cells produce 66-fold less α -factor than *MAT α* cells ($p=0.01$) (Figure 2.5). To measure the α -factor production of stimulated cells, we mixed the α -factor producing cells in a 10:1 mixture with *MAT α bar1 Δ* cells and measured the α -factor present in the supernatant. Stimulated **a**-playing- α cells produce only 20-fold less α -factor than stimulated wild-type *MAT α* cells ($p=9 \times 10^{-6}$) (Figure 2.5).

To test the effect of reduced pheromone production in *MAT α* cells, we knocked out *MF α 1*, which is the majority α -factor producer in *MAT α* cells²⁹. Using our assay, this manipulation results in a 12-fold reduction in α -factor production compared to wild-type *MAT α* cells in unstimulated cells ($p=0.02$), and a 9-fold reduction in α -factor production compared to wild-type *MAT α* cells in stimulated cells ($p=10^{-5}$) (Figure 2.5). We then compared the mating efficiency of *MAT α mfa1 Δ* cells, which have previously been shown to have decreased mating efficiency²⁹, to that of **a**-playing- α cells and determined that while there is a significant difference in the mating efficiency of *MAT α mfa1 Δ* cells and the mating efficiency of **a**-playing- α cells, *MAT α mfa1 Δ* cells only mate 1.5-fold more efficiently ($p=0.003$) (Figure 2.6). This led us to hypothesize that the reduced mating efficiency of the **a**-playing- α cells is due, at least in part, to low α -factor production.

To test this hypothesis, we increased α -factor production in the **a**-playing- α cells by placing the *MF α 1* gene under the control of the *TDH3* promoter. Although this promoter is not pheromone-regulated, it is one of the most highly expressed promoters in the yeast genome^{31, 32}, and thus, we expected to increase α -factor production to at least wild-type *MAT α* levels in the

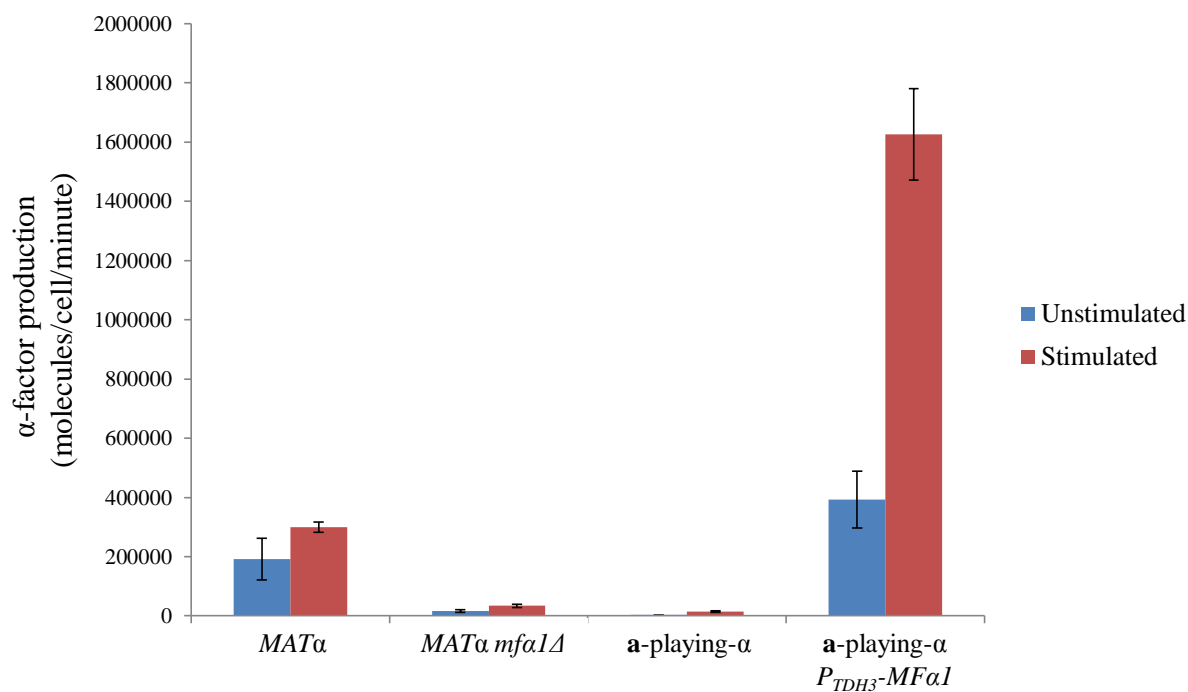


Figure 2.5. a-playing-α cells produce too little α-factor. α-factor production is measured by growing cells in YPD, harvesting the supernatant, and exposing *MATα bar1Δ* cells to the supernatant. The shmooing index of the *MATα bar1Δ* cells is measured and then compared to a standard curve to determine the concentration of α-factor present in the media. Error bars are standard deviation.

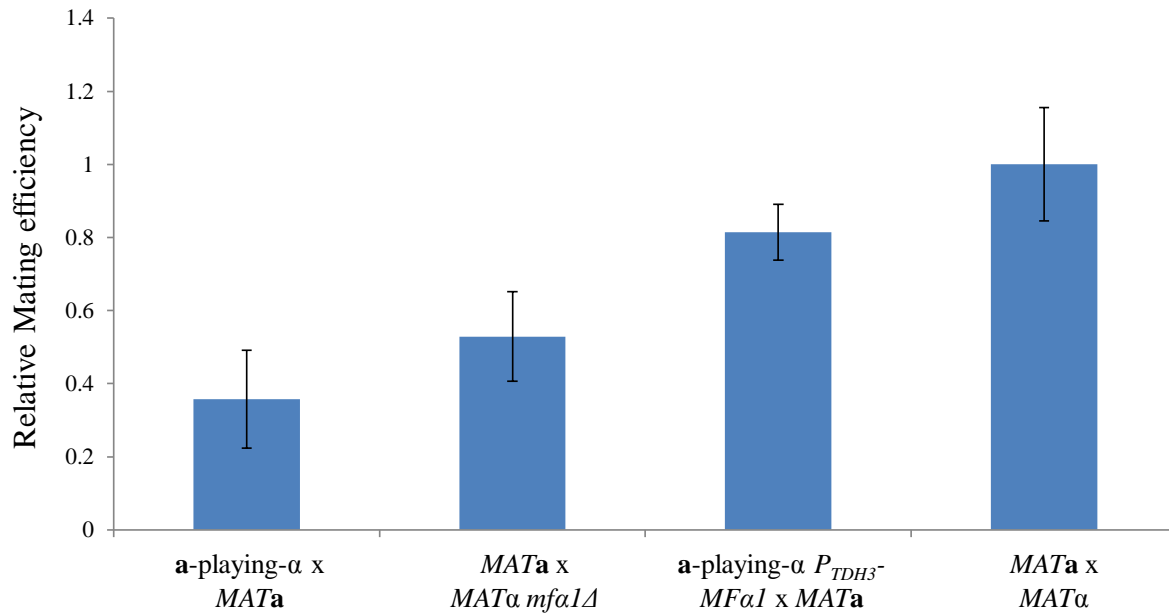


Figure 2.6. Increasing the α -factor production of a-playing- α cells increases mating efficiency. Mating efficiency of cells with different nutritional auxotrophies grown to log phase and then mixed at a 1:1 ratio on a nitrocellulose filter, which was placed on a YPD plate for 5 hours. Mating efficiencies are the percentage of diploids that form colonies on double dropout plates compared to the number of colonies formed on single dropout plates. Error bars are standard deviations.

a-playing- α cells. Unstimulated **a**-playing- α *P_{TDH3}-MF α 1* cells secrete twice as much α -factor as unstimulated *MAT α* cells ($p=0.04$), but when stimulated, **a**-playing- α *P_{TDH3}-MF α 1* cells secrete 5-fold more α -factor than stimulated *MAT α* cells ($p=10^{-4}$) (Figure 2.5). Since the *TDH3* promoter is not pheromone-regulated³³, this result suggests that α -factor production is at least in part regulated via post-translational mechanisms such as pheromone maturation and secretion. If low α -factor accounts for the low mating efficiency of the **a**-playing- α cells, we would expect the **a**-playing- α *P_{TDH3}-MF α 1* cells to have a mating efficiency approaching that of wild-type *MAT α* cells, which is indeed what we found (Figure 2.6). This corroborates previous results that sufficient α -factor production is important for efficient mating²⁹ and leads to two additional conclusions: i) there are no additional *MAT α* -specific genes beyond the ones we have manipulated that are detrimental to mating on solid media as a *MAT α* cell and ii) there are no additional *MAT α* -specific proteins beyond α -factor and Ste3 that are necessary for efficient mating on solid media as a *MAT α* cell.

AFB1 encodes a novel a-factor barrier protein

We next decided to look into the decreased mating efficiency of the α -playing-**a** cells. Because pheromone production is important for efficient mating^{29,30}, we investigated the **a**-factor production of the α -playing-**a** cells. Both α -factor and **a**-factor go through significant processing before secretion^{5,8}. However, while α -factor is secreted merely as a small peptide⁵, mature **a**-factor is prenylated, causing it to be very hydrophobic^{8,34}. The hydrophobicity of **a**-factor causes difficulties in biochemical quantification, so we measured the relative **a**-factor production of the α -playing-**a** cells by plating patches of **a**-factor producing cells and spraying supersensitive *MAT α* cells (*MAT α sst2 Δ* , which lack a GTPase activating protein that normally controls the life time of signaling from the pheromone-activated G protein³⁵⁻³⁸) over the patches in an **a**-factor

halo assay. Since supersensitive *MAT α* cells deleted for *SST2* arrest the cell cycle in response to low doses of **a**-factor^{36,37}, the **a**-factor produced by the cell patches will cause a halo with no cell growth to form around the **a**-factor-producing cell patches with a diameter that increases with the amount of **a**-factor produced by the cell patch (Figure 2.7A). The halo produced by the α -playing-**a** cells is smaller than that of wild-type *MATa* cells, implying that α -playing-**a** cells secrete less **a**-factor than wild-type *MATa* cells (Figure 2.7B).

Bar1 is a *MATa*-specific protease, which is secreted in order to cleave α -factor^{21,39}. Bar1 has two roles: it improves the accuracy of pheromone gradient detection, and it keeps the α -factor concentration at the surface of *MATa* cells low enough to avoid saturating the pheromone receptors⁴⁰⁻⁴². Biochemical evidence of a *MAT α* -specific, **a**-factor-cleaving endopeptidase has been reported⁴³, but the gene that encodes this endopeptidase has not been identified⁴⁴. It is easy to imagine that an **a**-factor barrier protein, such as an endopeptidase, would provide a similar service in improvement of gradient detection for *MAT α* cells and that secretion of such a protein by α -playing-**a** cells would cause lower net **a**-factor production and, thus, smaller halos. To test for the presence of a *MAT α* -specific **a**-factor barrier protein secreted from α -playing-**a** cells, we compared the halo sizes of a mixture of *MATa* cells with α -playing-**a** cells and a mixture of *MATa* cells with *MATa* cells that produce no **a**-factor. If the α -playing-**a** cells secrete an **a**-factor barrier protein, we would expect the halo size of the *MATa* cells mixed with α -playing-**a** cells to be smaller than that of the *MATa* cells mixed with *MATa mfa1 Δ mfa2 Δ* cells because the **a**-factor barrier protein would interfere with the **a**-factor from both the α -playing-**a** cells and the *MATa* cells. However, if there is no **a**-factor barrier protein, we would expect the halo size of the *MATa* cells mixed with α -playing-**a** cells to be larger than that of the *MATa* cells mixed with *MATa mfa1 Δ mfa2 Δ* cells because both the *MATa* cells and the α -playing-**a** cells are capable of

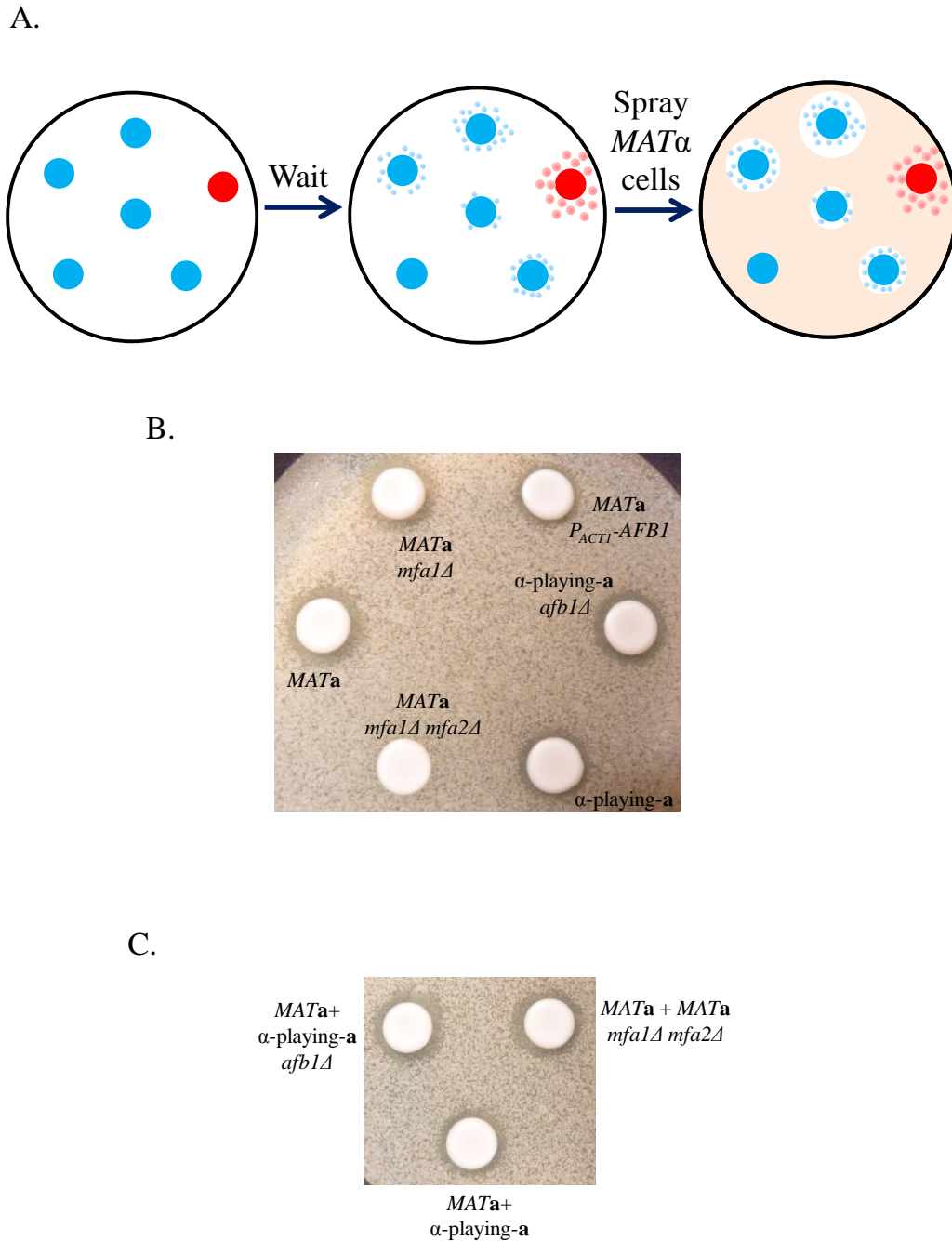


Figure 2.7. *AFB1* encodes a novel *a*-factor barrier protein. **A.** Halo assays are done by growing cell patches YPD overnight and then spraying super-sensitive $MAT\alpha$ *sst2Δ* cells over the cell patches. Where *a*-factor produced by the cell patches has diffused into the YPD, the $MAT\alpha$ *sst2Δ* cells will be unable to grow, forming a halo around the cell patch with a size that corresponds to the amount of *a*-factor secreted. **B.** Halo assays done on various cell patches containing a single cell type. $MAT\alpha$ *mfa1Δ* *mfa2Δ* is a negative control and $MAT\alpha$ is a positive control. **C.** Halo assays done on cell patches containing two cell types at a 1:8 ratio of $MAT\alpha$ cells to the experimental cell of interest.

secreting **a**-factor. When we observed the size of the halos produced by these two cell mixes, we saw that the halo produced by *MATa* cells mixed with α -playing-**a** cells is indeed smaller than the halo produced by *MATa* cells mixed with *MATa mfa1Δ mfa2Δ* cells, indicating that α -playing-**a** cells secrete an **a**-factor barrier protein (Figure 2.7C).

We decided to search for the gene responsible for this activity by comparing the transcriptomes of *MATa* and α -playing-**a** cells. Although microarrays and RNA sequencing have been done on pheromone stimulated *MATa* cells, similar experiments done on pheromone-stimulated *MAT α* cells have had to contend with the difficulties imposed by the extreme hydrophobicity of **a**-factor, which complicates quantification and, thus, direct comparison to *MATa* cells³³. Using the α -playing-**a** cells it is possible to easily and accurately investigate the transcriptome of pheromone stimulated cells that are *MAT α* at the *MAT* locus but are stimulated by **a**-factor. We used this unique opportunity and compared the transcriptomes of stimulated and unstimulated *MATa* and α -playing-**a** cells using RNA sequencing. We chose a concentration of pheromone, 10nM, in a regime in which *MATa bar1Δ* and α -playing-**a** cells with *BARI* under its endogenous promoter have a similar shmooing index (Figure 2.8A). By doing this we hoped to mitigate differences in gene expression that occur merely because of differences in the level of pheromone-induced signaling.

Just as *MAT α* cells do not secrete Bar1^{ref. 21}, we would not expect *MATa* cells to secrete an **a**-factor barrier protein. Thus, we hypothesized that a *MAT α* -specific **a**-factor barrier protein would be expressed more highly in both stimulated and unstimulated α -playing-**a** than stimulated and unstimulated *MATa* cells, respectively. 37 genes fit these parameters, and of these only one, *YLR040C*, is annotated as encoding for a secreted protein that is not already known to be important in mating in the Yeast Genome Database (<http://www.yeastgenome.org>) (Table 2.1).

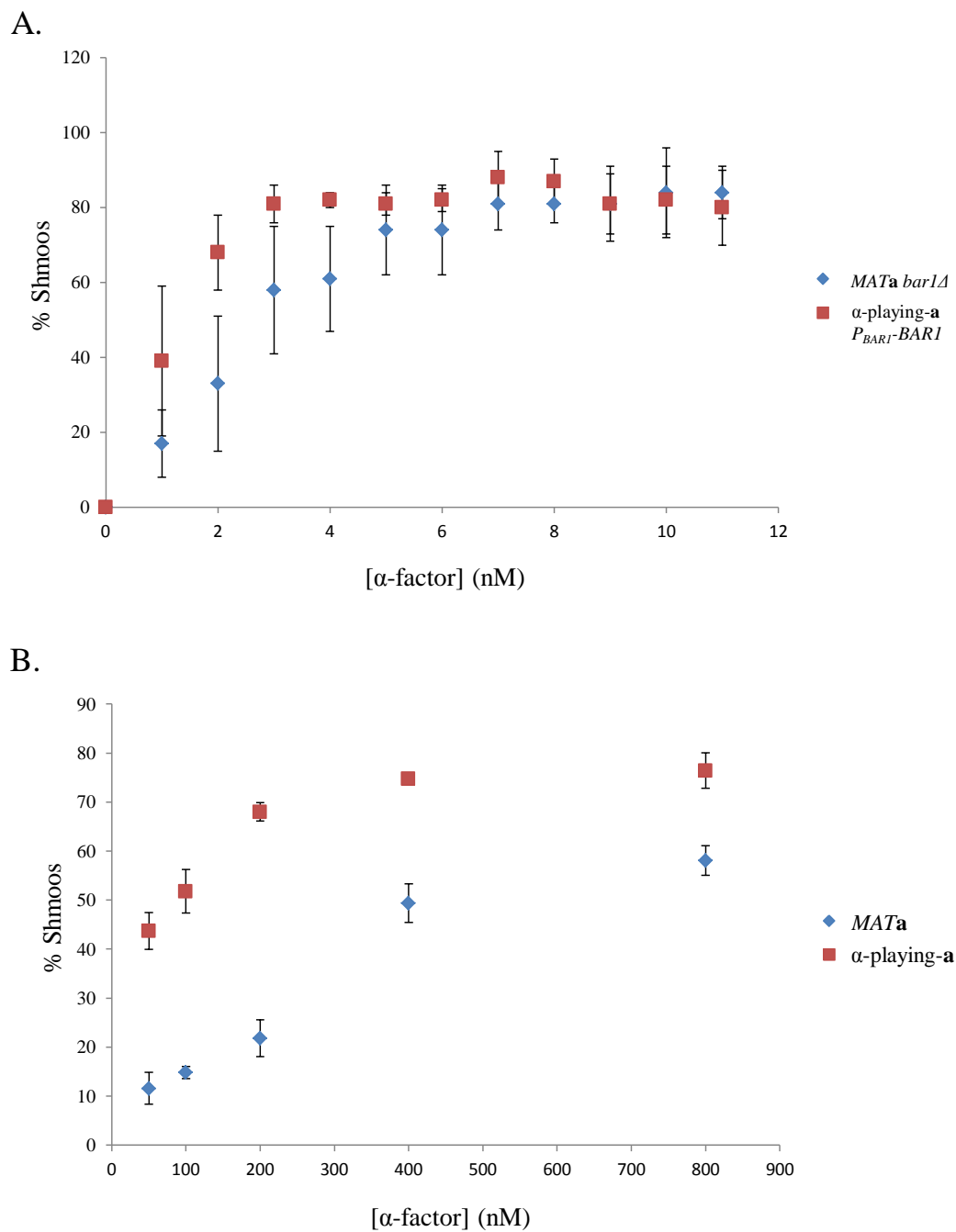


Figure 2.8. α -playing-a cells shmoos in response to α -factor. A. Shmoos indices of *MATa bar1Δ* cells and α -playing-a *P_{BARI}-BAR1* cells exposed to known concentrations of α -factor. Error bars are standard deviation. **B.** Shmoos indices of *MATa* cells and α -playing-a *P_{FUS1}*-BAR1* cells exposed to known concentrations of α -factor. Error bars are standard deviation.

Table 2.1. Genes identified as differentially expressed using RNA sequencing

Gene	Log ₂ Fold Change: Unstimulated <i>MATa</i> vs. Unstimulated α -playing-a	Log ₂ Fold Change: Stimulated <i>MATa</i> vs. Stimulated α -playing-a
<i>MFA2</i> *	-1.79769e+308	-1.79769e+308
<i>STE3</i> *	-1.79769e+308	-1.79769e+308
<i>HMRA1</i>	-1.79769e+308	-9.40214
<i>AGA2</i>	-7.28883	-9.28066
<i>BAR1</i> **	8.30734	9.11646
<i>ASG7</i>	-2.67534	-9.10911
<i>HMLα1</i>	7.5563	7.86148
<i>MATα1</i>	7.5563	7.86092
<i>YCR097W-A</i>	3.97042	6.96354
<i>MFA2</i>	-5.50844	-6.84593
<i>YDR008C</i>	4.29799	5.70204
<i>SAG1</i>	3.62891	5.49077
<i>YCL065W</i>	2.9083	5.33471
<i>TRP1</i>	4.34588	5.31384
<i>STE6</i>	-4.33495	-4.29995
<i>YNL146C-A</i>	-2.57191	-4.00106
<i>HMLα2</i>	1.28061	3.08194
<i>MATα2</i>	1.28061	3.08194
<i>MFA1</i>	-1.23469	-2.79786
<i>YLR040C</i>	3.40701	2.37516
<i>STE2</i>	-2.67485	-2.18172
<i>GYP8</i>	-1.02392	-2.05095
<i>YLR041W</i>	3.20998	2.03494
<i>ADE2</i>	-1.38094	-1.92617
<i>LEU1</i>	-2.47337	-1.82393
<i>OAC1</i>	-2.26803	-1.56826
<i>BAT1</i>	-1.69898	-1.19866
<i>ALD5</i>	-1.02533	-0.876709
<i>YML131W</i>	0.658035	0.690245

Table 2.1 (continued). Genes identified as differentially expressed using RNA sequencing

Gene	Log ₂ Fold Change: Unstimulated <i>MATa</i> vs. Unstimulated α -playing-a	Log ₂ Fold Change: Stimulated <i>MATa</i> vs. Stimulated α -playing-a
<i>FIT3</i>	-0.843601	-0.632751
<i>GDH1</i>	-0.866021	-0.600035
<i>ILV3</i>	-0.748211	-0.579364
<i>FSF1</i>	-0.639198	-0.575398
<i>BAP2</i>	-0.93542	-0.541877
<i>TPO2</i>	-0.741692	0.529846
<i>ADE17</i>	-0.747648	-0.4849
<i>YPR158C-D</i>	-0.614708	-0.480057
<i>YHR145C</i>	-0.606549	3.62557
<i>YCR041W</i>	2.13888	3.53217
<i>HMRA2</i>	0.311362	2.21746
<i>PCL1</i>	-0.121406	1.85386
<i>SNL1</i>	0.0524934	1.75104
<i>YLR346C</i>	1.1954	1.69724
<i>TOS6</i>	-0.0264883	1.30078
<i>INO1</i>	0.159527	1.29661
<i>PST1</i>	-0.0618079	1.15893
<i>OYE3</i>	-0.223013	1.0504
<i>TOS4</i>	-0.0364138	1.03405
<i>YOX1</i>	-0.116938	0.965281
<i>IMD2</i>	0.022238	0.954041
<i>PIR3</i>	0.466829	0.953975
<i>AXL1</i>	-0.519107	-0.930147
<i>LEU2</i>	0.212651	0.912745
<i>SPO11</i>	0.045965	0.881658
<i>MCD1</i>	0.0698717	0.869312
<i>HES1</i>	0.286167	0.845952
<i>FET3</i>	-0.320579	-0.829722
<i>THI22</i>	-0.261997	-0.813374

Table 2.1 (continued). Genes identified as differentially expressed using RNA sequencing

Gene	Log ₂ Fold Change: Unstimulated <i>MATa</i> vs. Unstimulated α -playing-a	Log ₂ Fold Change: Stimulated <i>MATa</i> vs. Stimulated α -playing-a
<i>GRE2</i>	0.126796	0.804999
<i>AAD16</i>	-0.00238782	0.802471
<i>YJL218W</i>	0.40768	0.790477
<i>YGL230C</i>	0.0462474	0.779615
<i>AAD6</i>	0.0279689	0.768583
<i>EGT2</i>	0.122072	0.757483
<i>YJR154W</i>	0.187072	0.737982
<i>MCH2</i>	0.349768	0.734259
<i>HTA2</i>	-0.171562	0.733893
<i>YDR134C</i>	0.0582465	0.691099
<i>PIR1</i>	-0.113912	0.689228
<i>KDX1</i>	0.516667	0.68283
<i>HSP31</i>	0.218705	0.675382
<i>YJR003C</i>	-0.152656	-0.664539
<i>YGR035C</i>	0.172402	0.664392
<i>APL1</i>	0.0679862	0.655452
<i>YNR064C</i>	0.0187628	0.639181
<i>YBR071W</i>	-0.0444652	0.629599
<i>HAL1</i>	0.475061	0.622606
<i>YOL159C</i>	0.0428331	0.622402
<i>CRG1</i>	0.223593	0.619031
<i>YNL134C</i>	-0.0355623	0.603511
<i>AAD10</i>	-0.146241	0.596282
<i>SVS1</i>	-0.0924168	0.589849
<i>SPI1</i>	0.43854	0.583315
<i>TAT1</i>	-0.349957	-0.573339
<i>HXK1</i>	-0.03119	-0.573261
<i>PRY2</i>	-0.286342	-0.567011
<i>YDR034W-B</i>	-0.507954	0.566863

Table 2.1 (continued). Genes identified as differentially expressed using RNA sequencing

Gene	Log ₂ Fold Change: Unstimulated <i>MATa</i> vs. Unstimulated α -playing-a	Log ₂ Fold Change: Stimulated <i>MATa</i> vs. Stimulated α -playing-a
<i>RNR1</i>	0.0733453	0.564782
<i>PDH1</i>	0.0823248	-0.563891
<i>SRL3</i>	0.07954	0.557634
<i>YNL058C</i>	0.213323	0.553972
<i>YKE4</i>	-0.0233873	0.543722
<i>ALT2</i>	-0.204164	-0.538135
<i>YPL257W-B</i>	-0.311592	-0.532874
<i>ACA1</i>	0.284868	0.522821
<i>HIS4</i>	-0.504345	-0.522659
<i>LEU9</i>	-0.223353	-0.521646
<i>ENA1</i>	-0.00247157	-0.51967
<i>PHO11</i>	-0.239362	-0.517299
<i>SIT1</i>	-0.460702	-0.506656
<i>YOL103W-B</i>	-0.314866	-0.503842
<i>PHO12</i>	-0.241366	-0.498986
<i>SSA4</i>	-0.102617	0.49795
<i>HHF1</i>	-0.0940667	0.497189
<i>DSE4</i>	0.293479	0.483196
<i>OAZ1</i>	0.130875	0.474892
<i>SEO1</i>	0.241226	0.47313
<i>NIS1</i>	-0.105821	0.469265
<i>HXT1</i>	-0.0823569	-0.466323
<i>YEH1</i>	0.0118294	0.463642
<i>RDN5-2</i>	2.89972	1.32504
<i>RDN5-4</i>	2.66498	1.02059
<i>RDN5-1</i>	2.34118	0.985198
<i>RDN5-6</i>	2.34118	0.985198
<i>RDN5-3</i>	2.5086	0.932077
<i>RDN5-5</i>	2.748	0.727355

Table 2.1 (continued). Genes identified as differentially expressed using RNA sequencing

Gene	Log ₂ Fold Change: Unstimulated <i>MATa</i> vs. Unstimulated α -playing- a	Log ₂ Fold Change: Stimulated <i>MATa</i> vs. Stimulated α -playing- a
<i>ARN2</i>	-0.652381	-0.409771
<i>FIT2</i>	-0.887553	-0.400677
<i>HMX1</i>	-0.637173	-0.323047
<i>YLR042C</i>	0.655711	0.309152
<i>ISU2</i>	-0.919987	-0.281347
<i>DIC1</i>	-1.25772	-0.28094
<i>GAP1</i>	-0.644093	-0.251608
<i>HAP4</i>	0.546667	0.228737
<i>YJL171C</i>	-0.590766	-0.199115
<i>DRE2</i>	-0.59276	-0.194585
<i>AGA1</i>	-0.850116	-0.154011
<i>HER1</i>	0.636144	0.151048
<i>MAE1</i>	-0.704876	-0.148065
<i>YGP1</i>	-1.03881	-0.0986889
<i>FUS1</i>	-0.653273	-0.0563373

Cells that are darker red are more highly expressed in α -playing-**a** *P_{BARI}-BARI* cells. Cells that are darker blue are more highly expressed in *MATa bar1Δ* cells. Cells outlined in bold lines indicate a statistically significant difference between the fragments per kilobase of transcript per million mapped reads (FPKM) of the expression of the gene in α -playing-**a** *P_{BARI}-BARI* and *MATa bar1Δ* cells ($p < 0.001$).

*Genes deleted in the α -playing-**a** cells.

**Genes deleted in the *MATa* cells.

YLR040C is uncharacterized, controlled by the transcription factor *Mat α 1*^{ref. 45}, shown to be translated by ribosome profiling⁴⁶, localizes to the cell wall^{47, 48}, and is expressed 5-fold higher in stimulated α -playing-**a** than *MATa* cells and 10-fold higher in unstimulated α -playing-**a** than *MATa* cells ($p < 10^{-6}$) (Table 2.1). The experiments described below demonstrate that *YLR040C* encodes an **a**-factor barrier protein, leading us to name this gene *AFB1* for **a**-factor barrier.

To determine whether *AFB1* is indeed the **a**-factor barrier protein, we knocked it out in the α -playing-**a** cells. The halos produced by α -playing-**a** *afb1 Δ* cells are larger than those of α -playing-**a** cells, indicating that more **a**-factor is secreted in α -playing-**a** cells lacking *AFB1* (Figure 2.7B). We also placed this gene under a strong (*ACT1*) promoter in *MATa* cells and observed a decrease in halo size compared to wild-type *MATa* cells, indicating that Afb1 is able to block **a**-factor secreted by *MATa* cells (Figure 2.7B). To test whether Afb1 is responsible for blocking **a**-factor produced by other cells, we made cell mixtures of *MATa* cells with α -playing-**a** *afb1 Δ* cells and compared the halo produced by this mixture to the halo produced by the mixtures of *MATa* cells with α -playing-**a** cells and to the halo produced by *MATa* cells with *MATa mfa1 Δ mfa2 Δ* cells. Corroborating our other results, the mixture of *MATa* cells with α -playing-**a** *afb1 Δ* cells has a larger halo than either the *MATa* cells with *MATa mfa1 Δ mfa2 Δ* cells or the *MATa* cells with α -playing-**a** cells, indicating that when Afb1 is not present in the cell mixtures, the **a**-factor from the wild-type *MATa* cells as well as that from the α -playing-**a** cells is free to interact with the super-sensitive *MAT α* cells (Figure 2.7C). Taken together our results are strong evidence that *AFB1* encodes for an **a**-factor barrier protein.

In 1989, Steden *et al.* isolated a mutant that caused supersensitivity to pheromone arrest in *MAT α* cells but not in *MATa* cells⁴⁹. This mutation did not affect the biochemical activity of the endopeptidase reported to be associated with *MAT α* cells⁴³ and was localized to the middle of

a 125 kilobase region on Chromosome XII, 608 kilobases away from *AFB1*^{ref. 49}. Thus, we do not believe that the mutation found by Steden *et al.*⁴⁹ was in *AFB1*. We cannot rule out the possibility that there is another protein with **a**-factor blocking activity, but we did not find a statistically significant difference in the gene expression of any of the 72 genes encoded in the region identified by Steden *et al.*⁴⁹ between *MATa* and α -playing-**a** cells (Table 2.2).

Although the expression of *AFB1* has an effect on the **a**-factor secretion of α -playing-**a** cells, we did not know whether *AFB1* was affecting the mating efficiency of α -playing-**a** cells. To test this, we crossed α -playing-**a** *afb1Δ* cells with wild-type *MATa* cells and observed a 4-fold increase in mating efficiency over α -playing-**a** cells ($p < 10^{-6}$) (Figure 2.9). However, when *AFB1* is deleted from wild-type *MATa* cells we do not see a significant decrease in mating efficiency (Figure 2.9). We hypothesized that this may be because small changes in **a**-factor production do not have a large effect on mating efficiency³⁰. To test this we deleted *MFA1* from wild-type *MATa* cells. We saw a small decrease in the halo size of *MATa mfa1Δ* as compared to wild-type *MATa* cells, but as previously reported³⁰, when we measured the mating efficiency of *MATa mfa1Δ* cells, we did not see a significant difference in mating efficiency from a wild-type cross (Figure 2.7B and 2.9).

α -playing-**a** cells shmoo but arrest only transiently in the presence of pheromone

Although the expression of *AFB1* in α -playing-**a** cells was responsible for a portion of the reduced mating efficiency of α -playing-**a** cells, α -playing-**a** *afb1Δ* cells crossed with *MATa* cells still mate 11-fold less efficiently than wildtype ($p < 10^{-6}$) (Figure 2.9). We hypothesized that the response of α -playing-**a** cells to pheromone could also be playing a role in the reduced mating efficiency of these cells. We first measured the shmooing index of α -playing-**a** cells with *BAR1* under its endogenous promoter when stimulated with known quantities of synthetic α -factor and

Table 2.2. Change in expression of genes in the region identified by Steden *et al.* 1989 between *MATa bar1Δ* and *P_{BARI}-BARI α*-playing-a cells

Gene	Log ₂ Fold Change: Stimulated <i>MATa</i> vs. Stimulated <i>α</i> -playing-a	Differentially expressed?	Log ₂ Fold Change: Unstimulated <i>MATa</i> vs. Unstimulated <i>α</i> -playing-a	Differentially expressed?
<i>ATG33</i>	0.1	no	-0.02	no
<i>RSC2</i>	-0.2	no	0.06	no
<i>YLR358C</i>	-0.03	no	0.04	no
<i>ADE13</i>	-0.08	no	-0.2	no
<i>VPS38</i>	0.1	no	-0.05	no
<i>DCR2</i>	-0.2	no	0.01	no
<i>YLR361C-A</i>	-0.002	no	-0.1	no
<i>STE11</i>	-0.2	no	0.06	no
<i>NMD4</i>	0.007	no	0.07	no
<i>YLR363W-A</i>	0.2	no	0.05	no
<i>GRX8</i>	0.2	no	0.06	no
<i>YLR365W</i>	1.3	no	-0.8	no
<i>YLR366W</i>	0.4	no	0.2	no
<i>YLR364C-A</i>	N/A	no	-0.2	no
<i>SNR44</i>	-0.2	no	-0.2	no
<i>RPS22B</i>	0.3	no	-0.3	no
<i>MDM30</i>	-0.08	no	0.06	no
<i>SSQ1</i>	-0.07	no	0.02	no
<i>ARC18</i>	0.06	no	-0.2	no
<i>ROM2</i>	-0.1	no	0.07	no
<i>SUR4</i>	-0.05	no	-0.1	no
<i>VID22</i>	-0.08	no	0.04	no
<i>YLR374C</i>	0.04	no	-0.2	no
<i>STP3</i>	0.02	no	-0.4	no
<i>PSY3</i>	0.3	no	0.03	no
<i>FBP1</i>	0.09	no	0.001	no
<i>SEC61</i>	-0.1	no	0.1	no
<i>YLR379W</i>	-0.2	no	0.1	no
<i>CSR1</i>	-0.02	no	0.2	no
<i>CTF3</i>	0.1	no	0.08	no
<i>NAM2</i>	-0.2	no	0.2	no
<i>SMC6</i>	-0.01	no	0.2	no
<i>IKI3</i>	-0.07	no	0.2	no
<i>SWC7</i>	0.09	no	0.4	no
<i>VAC14</i>	-0.1	no	0.2	no

Table 2.2 (continued). Change in expression of genes in the region identified by Steden *et al.* 1989 between *MATa bar1Δ* and *P_{BARI}-BARI α*-playing-a cells

Gene	Log ₂ Fold Change: Stimulated <i>MATa</i> vs. Stimulated <i>α</i> -playing-a	Differentially expressed?	Log ₂ Fold Change: Unstimulated <i>MATa</i> vs. Unstimulated <i>α</i> -playing-a	Differentially expressed?
<i>REH1</i>	-0.09	no	0.04	no
<i>RPS29A</i>	0.136	no	-0.3	no
<i>SNR34</i>	0.3	no	-0.3	no
<i>STE23</i>	-0.1	no	0.2	no
<i>ECM19</i>	-0.06	no	-0.06	no
<i>CCW14</i>	0.09	no	0.2	no
<i>ART10</i>	0.01	no	0.1	no
<i>ATP10</i>	-0.09	no	0.1	no
<i>CST9</i>	-0.1	no	0.08	no
<i>COX8</i>	0.2	no	-0.06	no
<i>VPS33</i>	-0.05	no	0.1	no
<i>AFG2</i>	-0.06	no	-0.01	no
<i>SKI2</i>	-0.1	no	0.2	no
<i>BDF1</i>	0.02	no	0.004	no
<i>DUS3</i>	-0.1	no	0.09	no
<i>YLR402W</i>	-0.3	no	0.2	no
<i>SFP1</i>	-0.1	no	0.1	no
<i>FLD1</i>	0.2	no	-0.06	no
<i>DUS4</i>	0.01	no	-0.007	no
<i>RPL31B</i>	0.014	no	-0.3	no
<i>YLR406C-A</i>	1.6	no	-2	no
<i>YLR407W</i>	0.1	no	-0.08	no
<i>BLS1</i>	0.1	no	0.09	no
<i>UTP21</i>	0.003	no	0.06	no
<i>VIP1</i>	-0.09	no	0.07	no
<i>YLR410W-A</i>	0	no	0	no
<i>YLR410W-B</i>	-0.3	no	-0.3	no
<i>CTR3</i>	0.04	no	-0.3	no
<i>BER1</i>	0.07	no	-0.08	no
<i>YLR412C-A</i>	N/A	no	3	no
<i>YLR413W</i>	0.03	no	0.3	no
<i>PUN1</i>	0.3	no	0.1	no
<i>YLR415C</i>	-0.1	no	0.4	no
<i>YLR416C</i>	0.1	no	0.4	no
<i>VPS36</i>	0.1	no	-0.2	no

Table 2.2 (continued). Change in expression of genes in the region identified by Steden *et al.* 1989 between *MATa bar1Δ* and *P_{BARI}-BARI α*-playing-a cells

Gene	Log ₂ Fold Change: Stimulated <i>MATa</i> vs. Stimulated <i>α</i> -playing-a	Differentially expressed?	Log ₂ Fold Change: Unstimulated <i>MATa</i> vs. Unstimulated <i>α</i> -playing-a	Differentially expressed?
<i>CDC73</i>	0.06	no	-0.1	no
<i>YLR419W</i>	-0.04	no	0.2	no

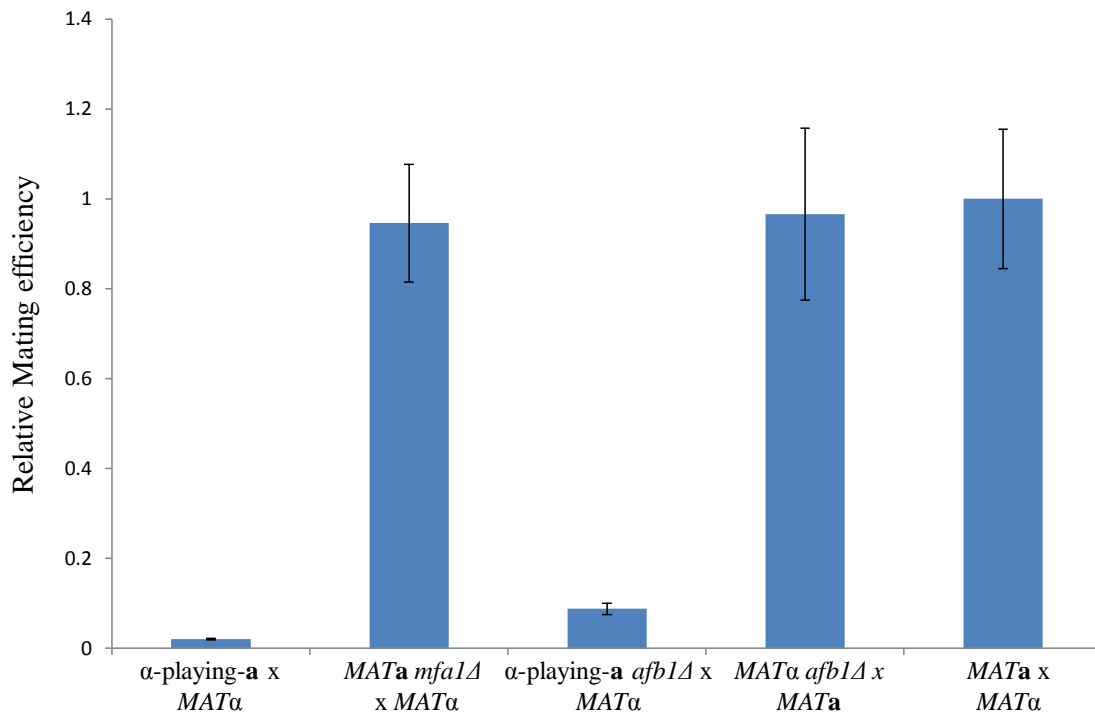


Figure 2.9. *AFB1* is partially responsible for the low mating efficiency of α -playing-a cells. Mating efficiency of cells with different nutritional auxotrophies grown to log phase and then mixed at a 1:1 ratio on a nitrocellulose filter, which was placed on a YPD plate for 5 hours. Relative mating efficiencies are the percentage of diploids that form colonies on double dropout plates compared to the number of colonies formed on single dropout plates relative to a wild-type cross. Error bars are standard deviations.

compared it to the shmooing index of *MATa bar1Δ* cells. We found that α -playing-**a** *P_{BAR1}-BAR1* cells are as sensitive to low concentrations of α -factor as *MATa* cells (Figure 2.8A).

In designing the transvestite strains, we attempted to choose promoters that would have similar patterns of expression to those of the genes in the wild-type cells by choosing the promoter of the reciprocal gene in the opposite mating type (e.g. expressing **a**-factor from the α -factor promoters in α -playing-**a** cells). However, when choosing a promoter for *BAR1* in the α -playing-**a** cells, we did not know of the reciprocal **a**-factor barrier protein, so we chose a mutant version of the *FUS1* promoter (which normally drives the expression of a gene that is required for cell fusion and is pheromone-induced in both *MATa* and *MATα* cells⁵⁰) that has a low basal level of expression and high stimulated level of expression²⁸, which we refer to as *P_{FUS1*}*. We measured the shmooing index of α -playing-**a** *P_{FUS1*}-BAR1* cells at various concentrations of α -factor and did two different comparisons (Figure 2.8B). The first was to compare the shmooing index of α -playing-**a** *P_{FUS1*}-BAR1* cells to α -playing-**a** *P_{BAR1}-BAR1* cells, which lack detectable Bar1 activity, to ask whether expression from the *FUS1** promoter produces active Bar1 (Figure 2.8). The α -playing-**a** *P_{FUS1*}-BAR1* cells make fewer shmoos at 50nM α -factor than α -playing-**a** *P_{BAR1}-BAR1* cells make at 2nM α -factor, indicating that α -playing-**a** *P_{FUS1*}-BAR1* cells are able to secrete Bar1 (p=0.02) (Figure 2.8). To ask if α -playing-**a** cells that express *BAR1* under *P_{FUS1*}* respond to α -factor like wild-type *MATa* cells, we compared the shmooing index of α -playing-**a** cells to wild-type *MATa* cells. The α -playing-**a** *P_{FUS1*}-BAR1* cells form a significantly higher percentage of shmoos at each concentration of α -factor tested than wild-type *MATa* cells (p<0.005) (Figure 2.8B). This suggests that these cells secrete less Bar1 than wild-type *MATa* cells. We wondered whether this decreased Bar1 secretion could account for the decreased mating efficiency of the α -playing-**a** cells. To test this possibility, we placed *BAR1* under the

*FUS1** promoter in wild-type *MATa* cells and determined that *MATa P_{FUS1*}-BAR1* cells are able to mate as efficiently as wild-type *MATa* cells when crossed to wild-type *MATα* cells (Figure 2.10). This implies that expressing *BAR1* under the *FUS1** promoter does not have a detrimental effect on mating efficiency at least when this is the sole manipulation to the mating machinery.

We also assayed the response of α -playing-**a** cells to α -factor in a microfluidics flow chamber that continuously supplies α -factor and allows us to monitor individual cells over time. Pheromone stimulation arrests *MATa* cells in G1 through phosphorylation of Far1, a protein that binds to cyclin-dependent kinase/cyclin complexes^{16, 51, 52}. When *MATa bar1Δ* cells are exposed to 10nM α -factor, their cell cycle remains arrested for many hours even though they form multiple successive shmoos (Figure 2.11A and Movie A1). However, even at this high concentration of α -factor, α -playing-**a** *bar1Δ* cells form shmoos but arrest only transiently (Figure 2.11B and Movie A2). Cells that do not fully arrest in response to pheromone stimulation have been shown to have decreased mating efficiency⁵³, so we hypothesize that the lack of sustained cell cycle arrest in pheromone-stimulated α -playing-**a** cells could contribute to their low mating efficiency.

The control of pheromone-induced cell cycle arrest in α -playing-a cells is complex

We hypothesized that the transient cell cycle arrest in α -playing-**a** cells is either due to a difference in the response of *MATα* and *MATa* cells to pheromone stimulation or to the interaction of Ste2 with *MATα*-specific genes in the α -playing-**a** cells. Our first step was to study the epistatic relationships of the genes involved in cell cycle arrest, so we manipulated the transcription factors expressed at the *MAT* locus. *MATα* cells express the transcription factors *Mata1*, which induces expression of α -specific genes, and *Mata2*, which represses expression of **a**-specific genes⁵⁴. The α -playing-**a** cells express these transcription factors but also express Ste2

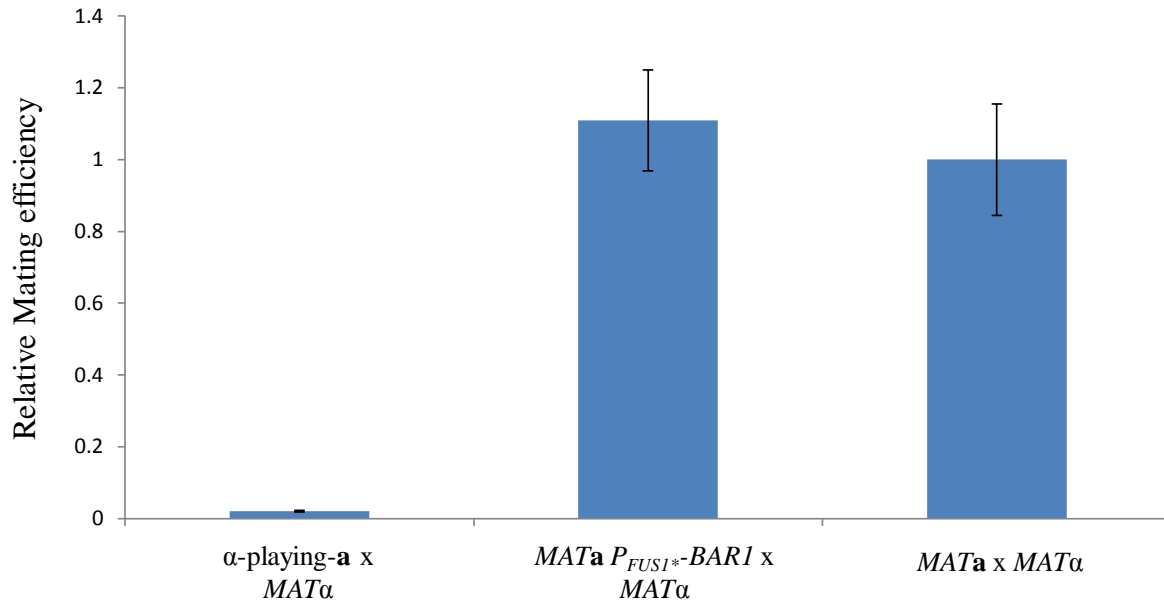
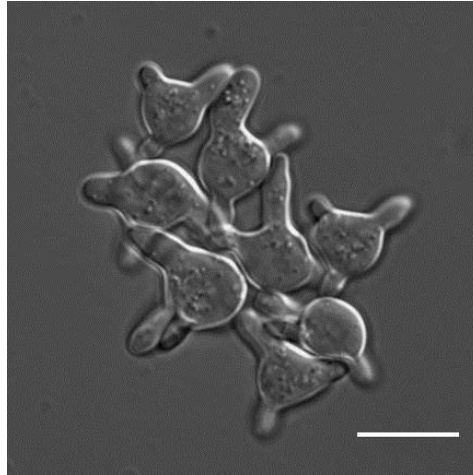


Figure 2.10. Expressing *BARI* under P_{FUSI^*} does not decrease the mating efficiency of $MATa$ cells. Mating efficiency of cells with different nutritional auxotrophies grown to log phase and then mixed at a 1:1 ratio on a nitrocellulose filter, which was placed on a YPD plate for 5 hours. Relative mating efficiencies are the percentage of diploids that form colonies on double dropout plates compared to the number of colonies formed on single dropout plates relative to a wild-type cross. Error bars are standard deviations.

A.



B.

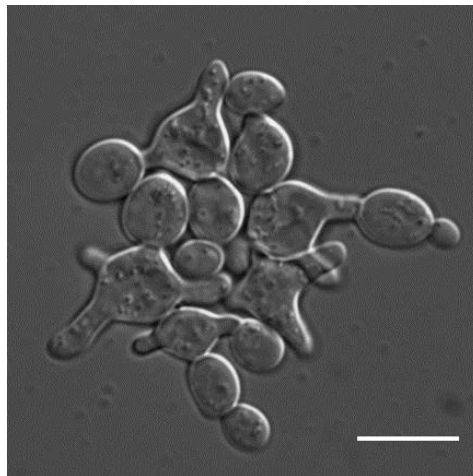
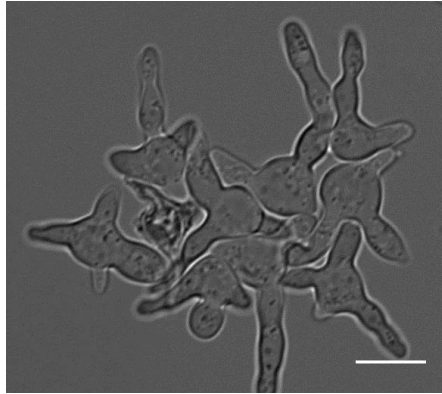


Figure 2.11. α -playing-a cells arrest only transiently in response to pheromone stimulation. **A.** *MATa bar1Δ* cells shmooing in continuously supplied SC+10nM α -factor in a microfluidics chamber. The picture was taken using DIC with 60x magnification 8 hours after the addition of α -factor. The scale bar indicates 10 μ m. **B.** α -playing-a *bar1Δ* cells shmooing and budding in continuously supplied SC+10nM α -factor in a microfluidics chamber. The picture was taken using DIC with 60x magnification 8 hours after the addition of α -factor. The scale bar indicates 10 μ m.

and **a**-factor. By deleting *MAT α 2* in α -playing-**a** cells, it is possible to create a strain that expresses all of the *MAT α* - and *MAT α* -specific genes but only the α -factor receptor, Ste2, and the pheromone, **a**-factor. When we expose the α -playing-**a** *bar1 Δ mata2 Δ* cells to α -factor, these cells are capable of sustained arrest, implying that the expression of *MAT α* -specific genes overrides the lack of prolonged arrest of α -playing-**a** cells (Figure 2.12A). An alternative manipulation, deletion of *MAT α 1* in *MAT α* cells, produces asexual haploid cells. The absence of *MAT α 1* prevents expression of α -specific genes, and the presence of *MAT α 2* prevents the expression of **a**-specific genes. We made such a strain responsive to α -factor by expressing Ste2 from the *HIS3* promoter, which normally leads to modest expression of an enzyme required for histidine synthesis^{55, 56}. When we expose *MAT α mata1 Δ P_{HIS3}-STE2* cells to α -factor, we observe that these cells also arrest indicating that the default for haploid cells exposed to pheromone is sustained G1 arrest (Figure 2.12B). This indicates that the interaction of the genes expressed in *MAT α* and *MAT α* cells involved in the control of cell cycle arrest is complex, leading us to hypothesize that multiple genes may control the lack of cell cycle arrest in α -playing-**a** cells.

To look for genes that might account for the lack of prolonged cell cycle arrest in α -playing-**a** cells, we compared the transcriptomes of *MAT α* and α -playing-**a** cells in the presence and absence of α -factor. We investigated four genes that were expressed at higher levels in α -playing-**a** cells than *MAT α* cells and were thus candidates for genes that had to be repressed to produce enduring cell cycle arrest: *PCL1*, *TOS4*, *SRL3*, and *YGR035C* (Table 2.1). We did not see any appreciable difference in the response to pheromone stimulation of α -playing-**a** cells deleted for these genes than the response to pheromone stimulation of α -playing-**a** wildtype for these genes, suggesting that none of them are individually responsible for the transient

A.



B.

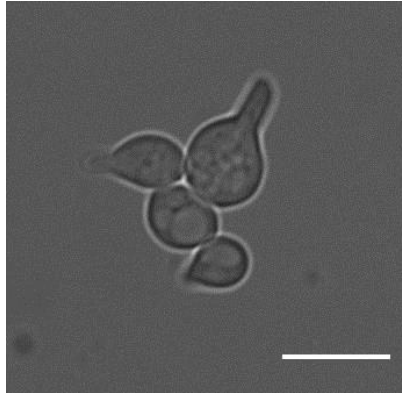


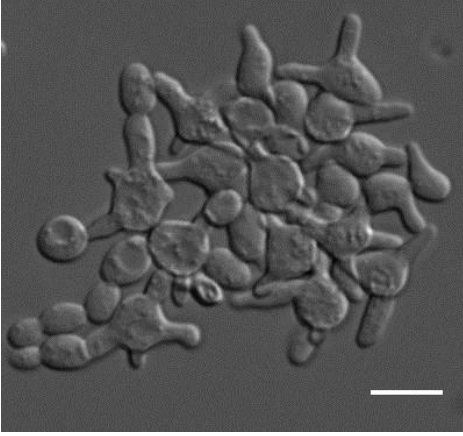
Figure 2.12. Manipulating the genes expressed at the *MAT* locus causes extended arrest in α -playing-a cells in response to pheromone stimulation. **A.** α -playing-a *bar1 Δ mata2 Δ* cells shmooing in continuously supplied SC+10nM α -factor in a microfluidics chamber. The picture was taken using brightfield with 60x magnification 8 hours after the addition of α -factor. The scale bar indicates 10 μ m. **B.** *MAT α mata1 Δ P_{HIS3}-STE2* cells shmooing in continuously supplied SC+10nM α -factor in a microfluidics chamber. The picture was taken using brightfield with 60x magnification 8 hours after the addition of α -factor. The scale bar indicates 10 μ m.

pheromone-induced arrest of α -playing-**a** cells (Figure 2.11B and 2.13A-D). We also investigated *GYP8*, which is expressed more strongly in *MATa* than in α -playing-**a** cells (Table 2.1). We deleted this gene from *MATa bar1Δ* cells and overexpressed it from the *ACT1* promoter in α -playing-**a** cells. We did not see a reproducible change in the response to either of these strains to α -factor when compared to the parent cells (Figure 2.11 and 2.13E-F). We hypothesize that the control of cell cycle arrest in α -playing-**a** cells is complex and involves multiple genes: either a combination of the genes that we manipulated individually or other genes whose differential transcription lies below the threshold that we set for our targeted genetic manipulations.

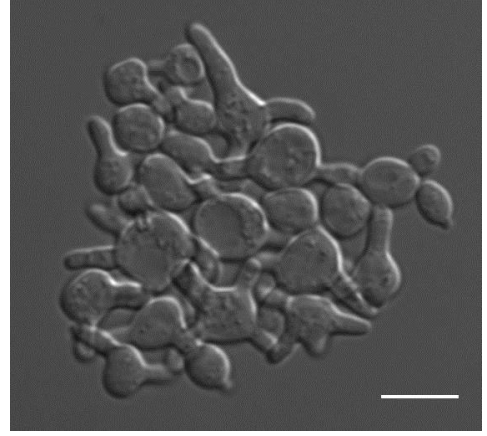
How robust is the mating response?

Investigating how efficiently the transvestite cells mate with wild-type partners is useful to learn more about the regulation of genes necessary in each mating type for efficient mating. Investigating how efficiently the transvestite cells mate with each other can tell us more about how robust the process of mating is. Crossing **a**-playing- α cells to wild-type *MATa* cells reduces mating efficiency three-fold, and crossing α -playing-**a** cells to wild-type *MATa* cells reduces mating 60-fold, relative to a wild-type *MATa* x *MATa* cross, but crossing the two transvestite strains resulted in a 650-fold decrease in mating efficiency compared to wildtype, leading us to believe that mating defects are synergistic ($p < 10^{-6}$) (Figure 2.14). If this synergism is largely due to reduced pheromone production by the transvestite strains, increasing pheromone production should increase the efficiency of the inter-transvestite cross. We increased α -factor production from **a**-playing- α by placing *MFa1* under the control of the *TDH3* promoter and **a**-factor production from α -playing-**a** cells by deleting *AFB1*. When these strains were mated to each other, they mate 90 times better than the cross between the parental **a**-playing- α and

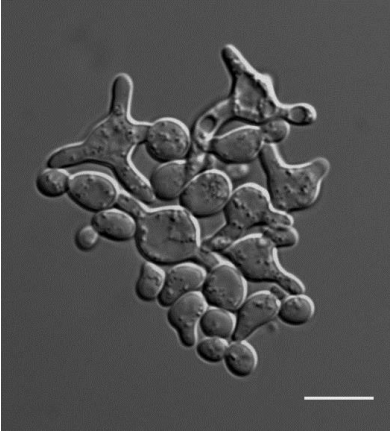
A.



B.



C.



D.

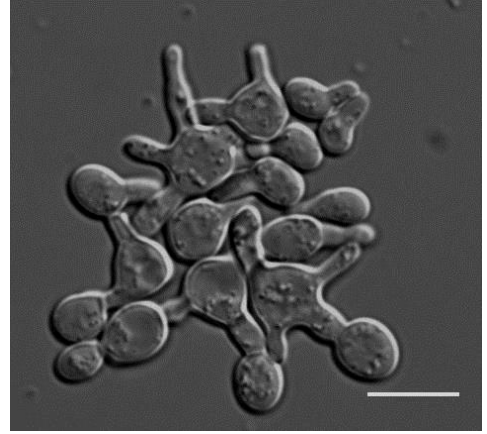
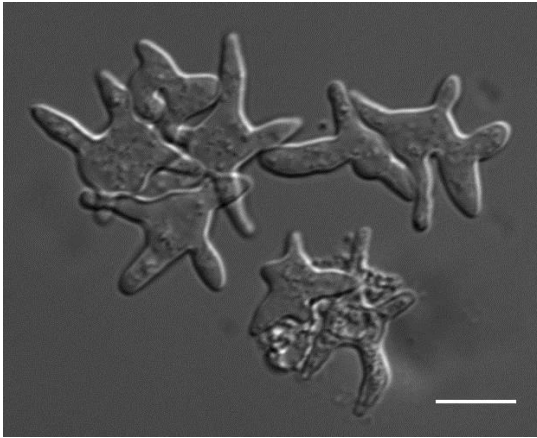


Figure 2.13. The control of pheromone-induced cell cycle arrest in α -playing-a cells is complex. **A.** α -playing-a *bar1Δ pcl1Δ* cells shmooing and budding in continuously supplied YPD+10nM α -factor in a microfluidics chamber. The picture was taken using DIC with 40x magnification 8 hours after the addition of α -factor. The scale bar indicates 10 μ m. **B.** α -playing-a *bar1Δ tos4Δ* cells shmooing and budding in continuously supplied SC+10nM α -factor in a microfluidics chamber. The picture was taken using DIC with 60x magnification 8 hours after the addition of α -factor. The scale bar indicates 10 μ m. **C.** α -playing-a *bar1Δ srl3Δ* cells shmooing and budding in continuously supplied SC+10nM α -factor in a microfluidics chamber. The picture was taken using DIC with 60x magnification 8 hours after the addition of α -factor. The scale bar indicates 10 μ m. **D.** α -playing-a *bar1Δ ygr035cΔ* cells shmooing and budding in continuously supplied SC+10nM α -factor in a microfluidics chamber. The picture was taken using DIC with 60x magnification 8 hours after the addition of α -factor. The scale bar indicates 10 μ m.

E.



F.

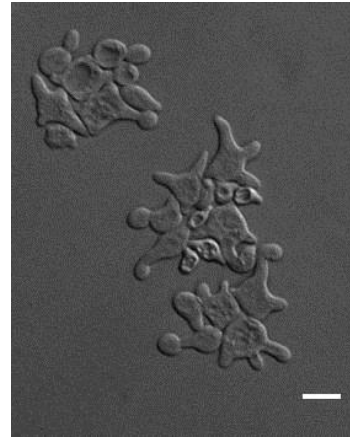


Figure 2.13 (continued). The control of pheromone-induced cell cycle arrest in α -playing-a cells is complex. E. *MATa bar1 Δ gyp8 Δ* cells shmooing in continuously supplied SC+10nM α -factor in a microfluidics chamber. The picture was taken using DIC with 60x magnification 8 hours after the addition of α -factor. The scale bar indicates 10 μ m. **F.** α -playing-a *bar1 Δ P_{ACT1}-GYP8* cells shmooing and budding in continuously supplied YPD+10nM α -factor in a microfluidics chamber. The picture was taken using DIC with 20x magnification 8 hours after the addition of α -factor. The scale bar indicates 10 μ m.

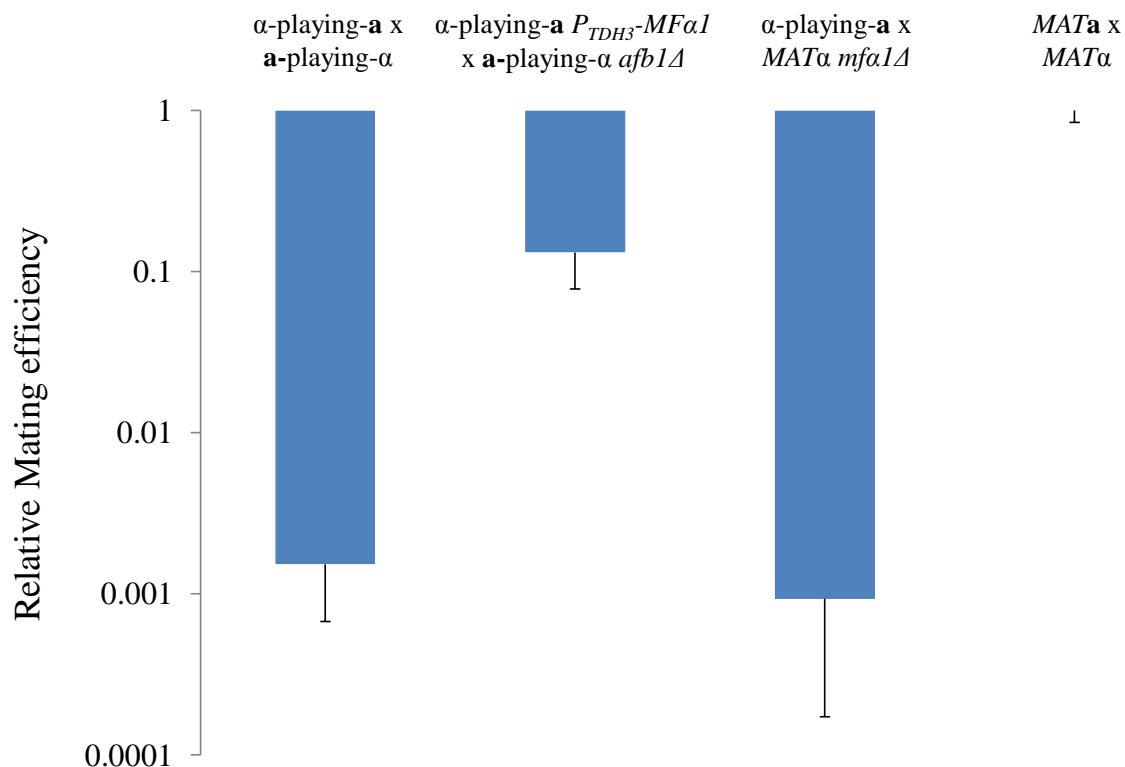


Figure 2.14. Mating is not robust to changes in pheromone production. Mating efficiency of cells with different nutritional auxotrophies grown to log phase and then mixed at a 1:1 ratio on a nitrocellulose filter, which was placed on a YPD plate for 5 hours. Relative mating efficiencies are the percentage of diploids that form colonies on double dropout plates compared to the number of colonies formed on single dropout plates relative to a wild-type cross. Error bars are standard deviations. Note the log scale.

α -playing-**a** cells ($p < 10^{-6}$) (Figure 2.14). Thus, improving pheromone production increases the mating frequency of the inter-transvestite cross to the point where it is only eight-fold less efficient than a standard *MATa* x *MAT α* cross ($p < 10^{-6}$) (Figure 2.14). It is possible that focusing on the production of and response to pheromone caused us to miss some of the mating defects caused by our genetic manipulations. However, we believe that at least in the case of the **a**-playing- α cells we have identified the main defect. To test this, we crossed the “pseudo”-**a**-playing- α cells (*MAT α mfa1 Δ*) with the α -playing-**a** cells. Since we hypothesize that low α -factor production is the main defect in the **a**-playing- α cells, we expect the mating efficiency of this cross to be similar to the transvestites crossed each other, and that is indeed what we see (Figure 2.14). This indicates that genetic manipulations that cause relatively modest mating defects in isolation, such as the deletion of one of the α -factor genes, can lead to much greater effects when combined with a compromised partner. We interpret this to mean that yeast have evolved a complex and highly regulated process to control cell fusion and that careful regulation of this process is important for efficient mating.

DISCUSSION

Mating type in yeast is controlled by the transcription factors expressed at the *MAT* loci³. *MATa*-specific genes have been well studied and consist of **a**-factor⁸, the **a**-factor exporter (Ste6)⁵⁷, the α -factor receptor (Ste2)⁹, a protease that cleaves α -factor (Bar1)²¹, a protein involved in attenuation of the pheromone response in early diploids (Asg7)^{26, 27}, and the **a**-agglutinin (Aga2)^{23, 45}. The genes specifically expressed by *MAT α* cells have been less well studied, but previously characterized *MAT α* -specific genes include α -factor^{5, 6}, the **a**-factor receptor (Ste3)⁷, and the α -agglutinin (Sag1)^{24, 45}. Because a useful way to study a system is to try to build it, we created transvestite strains that express the transcription factors at the *MAT* locus of one mating

type but the known mating type-specific genes of the opposite mating type. Although these transvestite strains are able to mate with wild-type strains of their original mating type and with each other, the mating efficiency of these crosses is lower than that of a wild-type cross indicating that there are additional mating type-specific genes that have not been fully characterized. We identified one of these, *AFBI*, as encoding an **a**-factor barrier protein. Our results indicate that mating is a fragile process: modest quantitative changes in the expression of critical genes reduce the efficiency of mating and multiple such defects interact synergistically to dramatically reduce mating efficiency.

MATa cells that express α -factor and Ste3 instead of **a**-factor and Ste2, which we call **a**-playing- α cells had a mating efficiency that was 3-fold less than that of wild-type *MATa* cells, and the mating defects in this strain were shown to be mainly from decreased α -factor production by **a**-playing- α cells. The level of α -factor secretion has previously been shown to be important for efficient mating²⁹, and by increasing the amount of α -factor secreted from the **a**-playing- α cells we were able to increase the mating efficiency of these cells to close to wild-type levels.

The mating defects associated with *MATa* cells that express **a**-factor and Ste2 instead of α -factor and Ste3, which we call α -playing-**a** cells, proved to be more complex. α -playing-**a** cells also showed decreased pheromone production when compared to wild-type *MATa* cells, and we were able to identify the function of the previously uncharacterized *MATa*-specific open reading frame, *YLR040C*, which we named *AFBI* for **a**-factor barrier. *AFBI* has been annotated as a secreted protein^{47,48} and we showed that the expression of this protein by **a**-factor-producing cells causes decreased **a**-factor secretion. Although there was no mating defect in wild-type *MATa* cells deleted for *AFBI*, as reported previously⁴⁵, we did see a significant increase in

mating efficiency when *AFB1* was deleted from the α -playing-**a** cells. We hypothesize that *AFB1* has two possible functions, which are not mutually exclusive.

The first is a reciprocal function to Bar1, which allows *MATa* cells to more accurately detect an α -factor gradient. While there are asymmetries in ascomycete mating, such as the difference in the hydrophobicity of **a**-factor and α -factor^{5,8}, gradient detection is important for both *MATa* cells searching for nearby, α -factor producing cells and for *MAT α* cells searching for nearby, **a**-factor producing cells⁴⁰. Modeling the effect of Bar1 in gradient detection by *MATa* cells has elucidated two general functions for Bar1: one for Bar1 that exits the cell wall of *MATa* cells⁴⁰ and another for Bar1 that is trapped in the cell wall of *MATa* cells^{41,42}.

Bar1 that exits the cell wall acts to decrease the lifetime of any given α -factor molecule, reducing the distance α -factor can travel and privileging the α -factor secreted from nearby cells for detection by a *MATa* cell⁴⁰. Meanwhile, Bar1 trapped in the cell wall of *MATa* cells serves as an α -factor sink, which both makes it easier for *MATa* cells to distinguish between two, close, potential *MAT α* partners by reducing the chance of saturating the pheromone receptors⁴² and makes it more likely that *MATa* cells will avoid each other and thus will end up choosing different *MAT α* partners⁴¹. Since α -factor is more hydrophilic than **a**-factor, it is likely to have a higher effective diffusion constant than **a**-factor, which binds tightly to hydrophobic surfaces. As a result, it is more likely that *MATa* cells will detect α -factor from distant *MAT α* cells than vice versa, making it less necessary for Afb1 to perform the reciprocal function to that accomplished by Bar1 that exits the cell wall. Accordingly, Afb1 is predicted to have a glycosylphosphatidylinositol (GPI) anchor⁴⁸, which would keep it associated with the plasma membrane. Thus, Afb1 is likely only to serve in distinguishing between two potential mating

partners and avoiding other *MATa* cells, and for this reason it is unsurprising that removing Bar1 from *MATa* cells causes a stronger mating defect than removing Afb1 from *MATa* cells.

The second possible function is that *AFB1* acts in concert with *ASG7* to reduce self-stimulation in early diploids^{26, 27}. Although translation of new mating-type specific genes is turned off in early diploids⁵⁴, these cells will still express both pheromones and both pheromone receptors until these proteins are either diluted out by cell growth or internalized and degraded. *Asg7* is an **a**-specific gene that interacts with Ste3 and causes internalization of Ste3 and Ste4, the G-protein β -subunit, in early diploids²⁶. It is possible that Afb1 sequesters or degrades the **a**-factor secreted by the early diploid and from nearby, unmated *MATa* cells in a dense mating mixture allowing the new diploid to form a first bud more rapidly.

We also observed that although α -playing-**a** *P_{BAR1}-BAR1* cells have shmooing indices that are statistically indistinguishable from wild-type *MATa bar1Δ* cells, α -playing-**a** *bar1Δ* cells only transiently arrest the cell cycle in response to pheromone stimulation. This is surprising because canonically it is thought that both *MATa* and *MATa* cells arrest the cell cycle in the same fashion: by signaling through Far1^{ref. 4, 51}. However, while cell cycle arrest in α -factor-stimulated *MATa* cells has been well studied, the hydrophobicity of **a**-factor has made the study of cell cycle arrest in **a**-factor-stimulated *MATa* cells more difficult. Using the α -playing-**a** cells, we were able to carefully study the effect of pheromone stimulation on α -playing-**a** cells over time, which allowed us to observe the transient cell cycle arrest of these cells. Once again we hypothesize that there are two possible explanations for the transient cell cycle arrest of α -playing-**a** cells. The first is that *MATa* and *MATa* cells do not respond to pheromone stimulation in the same way and that *MATa* cells shmoo but do not experience enduring arrest. However, this seems unlikely

because pheromone-stimulated arrest is important to ensure that nuclear fusion does not occur when the cells are in different phases of the cell cycle.

The second possibility is that the interaction between Ste2 and proteins present in the α -playing-**a** cells causes these cells to continue to cycle through the cell cycle, similar to the effect seen in *mata2 Δ ASG7* cells^{20, 27}. Unlike in the case of *ASG7*, the lack of sustained cell cycle arrest in α -playing-**a** cells is unlikely to be controlled by a single gene. By manipulating the transcription factors expressed at the *MAT* locus in α -playing-**a** and *MAT α* cells, we were able to determine that two different scenarios restore the ability of α -factor to cause prolonged cell cycle arrest: expressing *MAT α* -specific as well as *MAT α* -specific genes and expressing Ste2 in a cell that expresses neither *MAT α* -specific nor *MAT α* -specific genes. One interpretation of these results is that the default for cells expressing haploid specific genes is arrest and genes expressed in the α -playing-**a** cells are responsible for the lack of enduring arrest in response to α -factor stimulation. However, previous studies of *MAT α* -specific genes indicate that the only genes directly controlled by *Mata1* are *STE3*, *MF α 1*, *MF α 2*, *SAG1*, and *AFB1*^{ref. 45}, none of which are candidates for interfering with cell cycle arrest. We therefore investigated genes that might not have been identified in previous studies of unstimulated cells, focusing on those that are differentially expressed between pheromone-stimulated *MAT α* and α -playing-**a** cells. Although we tested the effect of deleting or overexpressing several genes individually in the α -playing-**a** cells, we were unable to detect a change in the response of these cells to α -factor stimulation, indicating either that we were unable to identify the gene responsible for this effect, or more likely, that complex regulation of multiple genes controls the response of *MAT α* cells expressing Ste2 to α -factor.

The strains engineered in this study provided a unique opportunity to investigate our knowledge of differences between *MATa* and *MAT α* cells. We have identified the function of a novel *a*-factor barrier protein as well as showing that complex expression of multiple genes may control cell cycle arrest in *MAT α* cells that express Ste2. Using the engineered transvestite strains we were able to compare the transcriptomes of pheromone-stimulated *MAT α* cells and *MATa* cells in a controlled manner. The addition of the knowledge gained in this study provides a more complete picture of the already well-studied pheromone response pathway in budding yeast.

MATERIALS and METHODS

Yeast strains and culturing

Strains used in this study are listed in Table 2.3. All strains were derived from the W303 wild-type background (*ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1*) using standard genetic techniques. All media was prepared as described in Sherman *et al.* 1974 and contained 2% wt/vol of glucose⁵⁸. Cells were either grown in Synthetic Complete media (2% glucose) (SC) or Yeast Extract Peptone Dextrose (2% glucose) (YPD) at 30°C in culture tubes on roller drums or agar plates or at room temperature (25°C) for timelapse microscopy. Mating assays were done on agar plates containing SC without adenine (SC-ade), SC without uracil (SC-ura), or SC without adenine and uracil (SC-ade-ura). Bovine serum albumin (BSA) was used to reduce the non-specific absorption of α -factor to glass and plastic surfaces. A 10% wt/vol stock was prepared in deionized water and then diluted into media to 0.1% wt/vol. Synthetic α -factor (Biosynthesis, Lewisville, TX) was suspended in dimethyl sulfoxide (DMSO) and then diluted into YPD + 0.1% BSA or SC + 0.1% BSA at the appropriate concentration. Peptone, yeast extract, and yeast nitrogen base were obtained from BD (VWR). Bacto-agar was obtained from

US Biological (Swampscott, MA). Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich.

Quantitative Mating Assay

Quantitative mating assays were modified from Reid and Hartwell, 1977⁵⁹. Briefly, cells were grown to log phase ($\sim 5 \times 10^6$ cells/mL). 5×10^6 cells were harvested from each strain, mixed at a 1:1 ratio, sonicated, and filtered onto a 0.22 μ m nitrocellulose filter (Millipore, MA). Filters were placed on a YPD plate and incubated at 30°C for 5 hours. To assay for the initial ratio of the haploid cells, a 2.5×10^{-5} dilution of the initial mating mixture was plated onto SC-ade and SC-ura plates. After 5 hours, cells were washed off the filters into 1 mL of deionized water and then plated onto SC-ade, SC-ura, and SC-ade-ura plates at appropriate dilutions to result in the growth of ~ 400 colonies per plate. SC dropout plates were incubated for 2 days before counting the colonies on each plate. Mating efficiencies were determined by dividing the number of colonies on the SC-ade-ura plate by the number of colonies on whichever of the SC-ade or SC-ura plate had fewer colonies. Three technical replicates were done of each mating assay and averaged for a single biological replicate. Error bars are standard deviation of biological replicates. Statistical significance was determined using Student's *t*-test.

Bioassay for α -factor production

The bioassay for α -factor production was modified from Goncalves-Sa and Murray, 2011⁶⁰. To measure unstimulated α -factor production, cells were grown to log phase ($\sim 5 \times 10^6$ cells/mL), washed into YPD + 0.1% BSA, and resuspended in BSA-coated culture tubes at 5×10^6 cells/mL in YPD + 0.1% BSA. The supernatant of the media was then harvested after a 15 minute, 30 minute, or 120 minute incubation at 30°C on a roller drum using BSA-coated 1 mL syringes (BD from VWR) and filtered through 0.45 μ m BSA-coated syringe filters (Pall

Corporation, New York). 5×10^5 log phase *MATa bar1Δ* cells were then incubated in 1x, 0.5x, and 0.2x dilutions of the supernatant for 2 hours, sonicated, fixed using 60% ethanol at 20°C, and resuspended into 20% glycerol in phosphate buffered saline (PBS). The percent of the cells shmooing was counted and compared to a standard curve.

Stimulated α -factor production was measured in the same way except that the cells being tested were grown to log phase ($\sim 5 \times 10^6$ cells/mL), counted, and washed into YPD + 0.1% BSA and 5×10^6 cells/mL of the cells being tested were mixed with 5×10^5 cells/mL *MATa bar1Δ* cells. After 2 hours incubation at 30°C on roller drums, the cells were washed into fresh YPD + 0.1% BSA and put into BSA-coated culture tubes. The supernatant was then harvested and analyzed as described above.

To generate a standard curve, *MATa bar1Δ* cells were incubated in integer values of synthetic α -factor concentrations between 0nM and 7nM and a best fit line was generated with an R^2 of 0.9 to determine a constant (K) for the relationship between α -factor concentration and the percentage of shmooos ($K=0.07$). α -factor production in molecules/cell/minute was then determined by $\frac{N \times K \times \% \text{ shmooos} \times \text{volume}}{r \times 2^D \times t \times l}$, where N is Avagadro's constant, r is the starting number of cells, D is the number of doublings expected, t is the incubation time, and l is the dilution. The expected number of doublings was calculated by the incubation time divided by an expected 90 minute doubling time for yeast.

Culture tubes and syringes were BSA-coated by incubating overnight at 4°C with PBS + 2% BSA. The PBS + 2% BSA was poured out immediately prior to use of the culture tube or syringe. Filters were BSA-coated by filtering 1mL of PBS + 2% BSA through them prior to use. At least 200 cells were counted to determine the percentage of cells shmooing. Error bars are standard deviation. Statistical significance was determined using Student's t -test.

Shmooing index

Cells were grown to log phase ($\sim 5 \times 10^6$ cells/mL), washed into YPD + 0.1% BSA with various concentrations of synthetic α -factor added at 5×10^5 cells/mL, and then incubated at 30°C on a roller drum for 2 hours. After incubation, the cells were sonicated, fixed using 60% ethanol at 20°C, and resuspended into 20% glycerol in PBS. At least 200 cells were counted to determine the percentage of cells shmooing. Error bars are standard deviation. Statistical significance was determined using Student's *t*-test.

Microscopy

Microscopy was done at room temperature using a Nikon Ti-E inverted microscope with either a 20x Plan Apo VC 0.75NC air lens, 40x Plan Fluor 0.75NA air lens, or a 60x Plan Apo VC 1.4NA oil lens, and images were acquired with a Photometrics CoolSNAP HQ camera (Roper Scientific, AZ). Timelapse photography was done using Metamorph 7.7 (Molecular Devices, CA).

Pictures of the *MATa/a* and *MAT α / α* diploids were acquired by growing cells to log phase ($\sim 5 \times 10^6$ cells/mL) at 30°C in YPD. Cells were then loaded into a microfluidics chamber (CellAsic, CA)⁶¹, which was pretreated by perfusing YPD through the chamber at 34kPa for 10 minutes. Once cells were loaded, YPD was perfused through the microfluidics chamber at 14kPa. Pictures were taken using a 60x Plan Apo VC 1.4NA oil lens (60x magnification) every 10 minutes for 10 hours using differential interference contrast with a 10ms exposure.

Pictures and movies of various strains responding to 10nM α -factor were done in a similar way. Cells were grown to log phase ($\sim 5 \times 10^6$ cells/mL) at 30°C in YPD, washed into rich media + 0.1% BSA (either YPD or SC as indicated in the figure legend), and loaded into a microfluidics chamber, which had been pretreated by perfusing PBS + 2% BSA through the

chamber at 34kPa for 10 minutes and then rich media + 0.1% BSA through the chamber at 34kPa for 10 minutes. Once the cells were loaded, rich media + 0.1% BSA + 10nM α -factor was perfused through the chamber at 14kPa. Pictures were taken every 10 minutes for 8 hours using either a 20x Plan Apo VC 0.75NC air lens (20x magnification), a 40x Plan Fluor 0.75NA air lens (40x magnification), or a 60x Plan Apo VC 1.4NA oil lens (60x magnification) with either differential interference contrast with a 10ms exposure or brightfield with a 6ms exposure.

For pictures of the **a**-playing- α cells responding to **a**-factor, *MATa P_{ACT1}-mCherry* cells were mixed with **a**-playing- α cells on a filter as described for the quantitative mating assay. Cells were washed off the filters into SC after 2.5 hours and placed directly onto glass slides (Corning). Pictures were taken immediately using a 20x Plan Apo VC 0.75NC air lens (20x magnification) with differential interference contrast with a 10ms exposure and fluorescence with a 300ms exposure.

Halo Assay

Halo assays were modified from Sprague, 1991⁶². Cells whose **a**-factor production was to be measured were grown to saturation in YPD at 30°C. For halo assays on individual cells, 4.5×10^8 cells of each strain were pelleted and resuspended in 20 μ L of deionized water. For halo assays on cell mixes, cells were mixed at a 1:8 ratio (*MATa* wildtype:cell type of interest) with a final cell count of 4.5×10^8 . Cells were pelleted and resuspended in 20 μ L of deionized water. 10 μ L of each strain or strain mix was spotted onto YPD plates and incubated overnight (~24 hours) at 30°C. Supersensitive *MATa sst2 Δ* cells grown to stationary phase were then sprayed over the cell spots using an Oenophilia martini atomizer (www.amazon.com). Plates were then incubated overnight (~18 hours) at 30°C and pictures were taken using a Panasonic Lumix DMC-TZ5 camera (www.panasonic.com).

RNA isolation and sequencing

Cells were grown to log phase (5×10^6 cells/mL) in YPD + 0.1% BSA at 30°C. 10mL of culture was harvested by spinning at 4°C, washed in 1mL RNase-free ice-cold water, pelleted, and flash frozen in dry ice. 10nM α -factor was added to the remaining culture, incubated for 2 hours at 30°C, and harvested in the same manner. RNA isolation was done as described in Collart and Oliveiro 2001⁶³ and suspended in sodium citrate. All necessary RNase-free chemicals were obtained from Invitrogen (NY) except for chloroform, which was obtained from VWR.

RNA libraries were prepared using the Illumina TruSEQ kit (www.illumina.com) and sequenced using an Illumina HiSeq 2000 with 50 base pair, single end reads with 89x mean coverage across the genome.

Sequence analysis

To analyze the sequencing data, the RNA sequences were aligned to the S288C reference genome r64 (downloaded from the Saccharomyces Genome Database www.yeastgenome.org) using TopHat⁶⁴. We then used Cufflinks to look for genes with significantly different levels of gene expression between *MATa bar1Δ* cells and α -playing-*a P_{BARI}-BARI* cells. Significant differences in expression were identified using the default setting in Cufflinks⁶⁵.

Table 2.3. Strains used in Chapter 2.

Strain Name	Genotype (all cells are in the W303 background)
LBHY29	<i>MATα P_{FUSI}-YFP @ LEU2 MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY41	<i>MATα ade2-1 can1-100 his3-11,15 leu2-112 trp1-1</i>
LBHY44	<i>MATα P_{FUSI}-YFP @ LEU2 bar1Δ::HphMX4 MFα1-HIS3::mfa1Δ MFα2-TRP1::mfa2Δ STE3-NatMX4::ste2Δ asg7Δ::URA3(<i>Kluyveromyces lactis</i>) ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY47	<i>MATα P_{FUSI}-YFP @ LEU2 P_{MFα1}-STE6-URA3 MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ P_{FUSI}*-BAR1-KanMX6 ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY49	<i>MATα P_{FUSI}-YFP @ LEU2 PMFα1-STE6-ura3⁻ MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ P_{FUSI}*-BAR1-KanMX6 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY89	<i>MATα mfa1Δ::NatMX4 ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1</i>
LBHY92	<i>MATα mfa1Δ::NatMX4 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1</i>
LBHY93	<i>MATα mfa1Δ::NatMX4 ade2-1 can1-100 his3-11,15 leu2-112 trp1-1</i>
LBHY98	<i>MATα P_{FUSI}-YFP @ LEU2 bar1Δ::HphMX4 MFα1-HIS3::mfa1Δ MFα2-TRP1::mfa2Δ STE3-NatMX4::ste2Δ asg7Δ::URA3(<i>K. lactis</i>) PACT1-yCerulean @ ADE2 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY108	<i>MATα PACT1-mCherry-HIS3MX6 @ PACT1 ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1</i>
LBHY156	<i>MATα mfa1Δ::KanMX6 mfa2Δ::HphMX4 ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1</i>
LBHY177	<i>MATα P_{FUSI}-YFP @ LEU2 MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ bar1Δ::KanMX6 ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY261	<i>MATα mata1Δ::NatMX4 hmlα1Δ::URA3 (<i>Candida albicans</i>) P_{HIS3}-STE2-KanMX6</i>
LBHY286	<i>MATα mfa1Δ::KanMX6 ade2-1 can1-100 his3-11,15 leu2-112 trp1-1</i>
LBHY290	<i>MATα P_{FUSI}*-BAR1-KanMX6 ade2-1 can1-100 his3-11,15 leu2-112 trp1-1</i>
LBHY316	<i>MATα/a P_{FUSI}-YFP @ LEU2 P_{MFα1}-STE6-URA3/P_{STE6}-STE6 MFA1-TRP1::mfa1Δ/MFα1 MFA2-HIS3::mfa2Δ/MFα2 STE2-NatMX4::ste3Δ/STE3 P_{FUSI}*-BAR1-KanMX6/P_{BAR1}-BAR1 ADE2/ade2-1 can1-100 /can1-100 his3-11,15/his3-11,15 trp1-1/trp1-1 ura3-1/ura3-1</i>
LBHY318	<i>MATα/a P_{FUSI}-YFP @ LEU2 bar1Δ::HphMX4/BAR1 MFα1-HIS3::mfa1Δ/MFα1 MFα2-TRP1::mfa2Δ/MFA2 STE3-NatMX4::ste2Δ/STE2 asg7Δ::URA3(<i>K. lactis</i>)/ASG7 ADE2/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 trp1-1/trp1-1 ura3-1/ura3-1</i>
LBHY346	<i>MATα P_{FUSI}-YFP @ LEU2 P_{MFα1}-STE6-URA3 MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ P_{FUSI}*-BAR1-KanMX6 afb1Δ::HphMX4 ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY350	<i>MATα afb1Δ::HphMX4 ade2-1 can1-100 his3-11,15 leu2-112 trp1-1</i>
LBHY352	<i>MATα afb1Δ::HphMX4 ade2-1 can1-100 his3-11,15 leu2-112 trp1-1</i>

Table 2.3 (continued). Strains used in Chapter 2.

Strain Name	Genotype (all cells are in the W303 background)
LBHY356	<i>MATa bar1Δ::ADE2 HIS3:SPA2-YFP gyp8Δ::HphMX4 ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1</i>
LBHY358	<i>MATα P_{FUS1}-YFP @ LEU2 MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ bar1Δ::KanMX6 pcl1Δ::HphMX4 ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY360	<i>MATα P_{FUS1}-YFP @ LEU2 MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ bar1Δ::KanMX6 ygr035cΔ::HphMX4 ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY364	<i>MATα P_{FUS1}-YFP @ LEU2 MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ bar1Δ::KanMX6 tos4Δ::HphMX4 ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY366	<i>MATα P_{FUS1}-YFP @ LEU2 MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ bar1Δ::KanMX6 srl3Δ::HphMX4 ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY395	<i>MATa P_{FUS1}-YFP @ LEU2 bar1Δ::HphMX4 Mfa1-HIS3::mfa1Δ Mfa2-TRP1::mfa2Δ STE3-NatMX4::ste2Δ asg7Δ::URA3(K. lactis) P_{TDH3}-Mfa1:KanMX6 @ P_{TDH3} ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY397	<i>MATa P_{ACT1}-AFB1:KanMX6 @ P_{ACT1} ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1</i>
LBHY403	<i>MATα P_{FUS1}-YFP @ LEU2 MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ bar1Δ::KanMX6 mata2a::HphMX4 hmla2Δ::URA3 (C. albicans) ade2-1 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY406	<i>MATα P_{FUS1}-YFP @ LEU2 MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ bar1Δ::KanMX6 P_{ACT1}-GYP8 @ ADE2 can1-100 his3-11,15 trp1-1 ura3-1</i>
LBHY410	<i>MATα P_{FUS1}-YFP @ LEU2 P_{Mfa1}-STE6-ura3⁻ MFA1-TRP1::mfa1Δ MFA2-HIS3::mfa2Δ STE2-NatMX4::ste3Δ P_{FUS1}*-BAR1-KanMX6 afb1Δ::HphMX4 can1-100 his3-11,15 trp1-1 ura3-1</i>
MP 381	<i>MATα bar1Δ::ADE2 SPA2-YFP:HIS3 ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1</i>
MP 384	<i>MATa bar1Δ::ADE2 SPA2-YFP:HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>
MP 420	<i>MATa SPA2-CFP:KanMX6 P_{FUS1}-YFP @ HIS3 can1-100 leu2-112 trp1-1 ura3-1</i>
SLY412	<i>MATa ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1 (W303 wildtype)</i>
SLY413	<i>MATα ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1 (W303 wildtype)</i>

References

1. Madhani, H. D. in *From a to [alpha] : yeast as a model for cellular differentiation* 115 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2007).
2. Ydenberg, C. A. & Rose, M. D. Yeast mating: a model system for studying cell and nuclear fusion. *Methods Mol. Biol.* **475**, 3-20 (2008).
3. Haber, J. E. Mating-type gene switching in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **32**, 561-599 (1998).
4. Bardwell, L. A walk-through of the yeast mating pheromone response pathway. *Peptides* **26**, 339-350 (2005).
5. Kurjan, J. & Herskowitz, I. Structure of a yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor. *Cell* **30**, 933-943 (1982).
6. Singh, A. *et al.* *Saccharomyces cerevisiae* contains two discrete genes coding for the alpha-factor pheromone. *Nucleic Acids Res.* **11**, 4049-4063 (1983).
7. Hagen, D. C., McCaffrey, G. & Sprague, G. F., Jr. Evidence the yeast STE3 gene encodes a receptor for the peptide pheromone a factor: gene sequence and implications for the structure of the presumed receptor. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 1418-1422 (1986).
8. Betz, R. & Duntze, W. Purification and partial characterization of a factor, a mating hormone produced by mating-type-a cells from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **95**, 469-475 (1979).
9. Blumer, K. J., Reneke, J. E. & Thorner, J. The STE2 gene product is the ligand-binding component of the alpha-factor receptor of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **263**, 10836-10842 (1988).
10. Burkholder, A. C. & Hartwell, L. H. The yeast α -factor receptor: structural properties deduced from the sequence of the STE2 gene. *Nucleic Acids Res.* **13**, 8463-8475 (1985).
11. Hartig, A., Holly, J., Saari, G. & MacKay, V. L. Multiple regulation of STE2, a mating-type-specific gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **6**, 2106-2114 (1986).
12. Fields, S., Chaleff, D. T. & Sprague, G. F., Jr. Yeast STE7, STE11, and STE12 genes are required for expression of cell-type-specific genes. *Mol. Cell. Biol.* **8**, 551-556 (1988).
13. Zeitlinger, J. *et al.* Program-specific distribution of a transcription factor dependent on partner transcription factor and MAPK signaling. *Cell* **113**, 395-404 (2003).
14. Dohlman, H. G. & Thorner, J. W. Regulation of G protein-initiated signal transduction in yeast: paradigms and principles. *Annu. Rev. Biochem.* **70**, 703-754 (2001).

15. Johnson, G. L. & Lapadat, R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298**, 1911-1912 (2002).
16. Chang, F. & Herskowitz, I. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. *Cell* **63**, 999-1011 (1990).
17. Butty, A. C., Pryciak, P. M., Huang, L. S., Herskowitz, I. & Peter, M. The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. *Science* **282**, 1511-1516 (1998).
18. Segall, J. E. Polarization of yeast cells in spatial gradients of alpha mating factor. *Proceedings of the National Academy of Sciences* **90**, 8332-8336 (1993).
19. Casamayor, A. & Snyder, M. Bud-site selection and cell polarity in budding yeast. *Curr. Opin. Microbiol.* **5**, 179-186 (2002).
20. Bender, A. & Sprague, G. Pheromones and pheromone receptors are the primary determinants of mating specificity in the yeast *Saccharomyces cerevisiae*. *Genetics* **121**, 463-476 (1989).
21. Sprague Jr, G. F. & Herskowitz, I. Control of yeast cell type by the mating type locus: I. Identification and control of expression of the a-specific gene BAR1. *J. Mol. Biol.* **153**, 305-321 (1981).
22. Jackson, C. L. & Hartwell, L. H. Courtship in *S. cerevisiae*: both cell types choose mating partners by responding to the strongest pheromone signal. *Cell* **63**, 1039-1051 (1990).
23. Cappellaro, C. *et al.* *Saccharomyces cerevisiae* a- and alpha-agglutinin: characterization of their molecular interaction. *EMBO J.* **10**, 4081 (1991).
24. Doi, S., Tanabe, K., Watanabe, M., Yamaguchi, M. & Yoshimura, M. An α -specific gene, SAG1 is required for sexual agglutination in *Saccharomyces cerevisiae*. *Curr. Genet.* **15**, 393-398 (1989).
25. Roy, A., Lu, C., Marykwas, D., Lipke, P. & Kurjan, J. The AGA1 product is involved in cell surface attachment of the *Saccharomyces cerevisiae* cell adhesion glycoprotein a-agglutinin. *Mol. Cell. Biol.* **11**, 4196-4206 (1991).
26. Kim, J. *et al.* Localization and Signaling of Gbeta Subunit Ste4p Are Controlled by a-Factor Receptor and the a-Specific Protein Asg7p. *Science Signaling* **20**, 8826 (2000).
27. Roth, A. F., Nelson, B., Boone, C. & Davis, N. G. Asg7p-Ste3p inhibition of pheromone signaling: regulation of the zygotic transition to vegetative growth. *Science Signaling* **20**, 8815 (2000).

28. Ingolia, N. T. & Murray, A. W. Positive-feedback loops as a flexible biological module. *Curr. Biol.* **17**, 668-677 (2007).
29. Kurjan, J. Alpha-factor structural gene mutations in *Saccharomyces cerevisiae*: effects on alpha-factor production and mating. *Mol. Cell. Biol.* **5**, 787-796 (1985).
30. Michaelis, S. & Herskowitz, I. The a-factor pheromone of *Saccharomyces cerevisiae* is essential for mating. *Mol. Cell. Biol.* **8**, 1309-1318 (1988).
31. Krebs, E. G. Yeast glyceraldehyde-3-phosphate dehydrogenase I. Electrophoresis of fractions precipitated by nucleic acid. *J. Biol. Chem.* **200**, 471-478 (1953).
32. McAlister, L. & Holland, M. J. Differential expression of the three yeast glyceraldehyde-3-phosphate dehydrogenase genes. *J. Biol. Chem.* **260**, 15019 (1985).
33. Roberts, C. J. *et al.* Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles. *Science Signaling* **287**, 873 (2000).
34. Chen, P., Sapperstein, S. K., Choi, J. D. & Michaelis, S. Biogenesis of the *Saccharomyces cerevisiae* mating pheromone a-factor. *J. Cell Biol.* **136**, 251-269 (1997).
35. Apanovitch, D. M., Slep, K. C., Sigler, P. B. & Dohlman, H. G. Sst2 is a GTPase-activating protein for Gpa1: purification and characterization of a cognate RGS-G α protein pair in yeast. *Biochemistry (N. Y.)* **37**, 4815-4822 (1998).
36. Chan, R. K. & Otte, C. A. Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by a factor and alpha factor pheromones. *Mol. Cell. Biol.* **2**, 11-20 (1982).
37. Chan, R. K. & Otte, C. A. Physiological characterization of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by a factor and alpha factor pheromones. *Mol. Cell. Biol.* **2**, 21 (1982).
38. Dohlman, H. G., Song, J., Ma, D., Courchesne, W. E. & Thorner, J. Sst2, a negative regulator of pheromone signaling in the yeast *Saccharomyces cerevisiae*: expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein alpha subunit). *Mol. Cell. Biol.* **16**, 5194-5209 (1996).
39. MacKay, V. L. *et al.* The *Saccharomyces cerevisiae* BAR1 gene encodes an exported protein with homology to pepsin. *Proceedings of the National Academy of Sciences* **85**, 55-59 (1988).
40. Barkai, N., Rose, M. D. & Wingreen, N. S. Protease helps yeast find mating partners. *Nature* **396**, 422-423 (1998).
41. Jin, M. *et al.* Yeast dynamically modify their environment to achieve better mating efficiency. *Science signaling* **4**, ra54 (2011).

42. Rappaport, N. & Barkai, N. Disentangling signaling gradients generated by equivalent sources. *J. Biol. Phys.* **38**, 267-278 (2012).
43. Marcus, S., Xue, C., Naider, F. & Becker, J. Degradation of a-factor by a *Saccharomyces cerevisiae* alpha-mating-type-specific endopeptidase: evidence for a role in recovery of cells from G1 arrest. *Mol. Cell. Biol.* **11**, 1030-1039 (1991).
44. Michaelis, S. & Barrowman, J. Biogenesis of the *Saccharomyces cerevisiae* Pheromone a-Factor, from Yeast Mating to Human Disease. *Microbiology and Molecular Biology Reviews* **76**, 626-651 (2012).
45. Galgoczy, D. J. *et al.* Genomic dissection of the cell-type-specification circuit in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences* **101**, 18069-18074 (2004).
46. Brar, G. A. *et al.* High-resolution view of the yeast meiotic program revealed by ribosome profiling. *Science* **335**, 552-557 (2012).
47. Giaever, G. *et al.* Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**, 387-391 (2002).
48. Hamada, K., Terashima, H., Arisawa, M., Yabuki, N. & Kitada, K. Amino acid residues in the ω -minus region participate in cellular localization of yeast glycosylphosphatidylinositol-attached proteins. *J. Bacteriol.* **181**, 3886-3889 (1999).
49. Steden, M., Betz, R. & Duntze, W. Isolation and characterization of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by the mating hormone a-factor. *Molecular and General Genetics MGG* **219**, 439-444 (1989).
50. Trueheart, J., Boeke, J. D. & Fink, G. R. Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. *Mol. Cell. Biol.* **7**, 2316-2328 (1987).
51. Peter, M. & Herskowitz, I. Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. *Science* **265**, 1228-1231 (1994).
52. Tyers, M. & Futcher, B. Far1 and Fus3 link the mating pheromone signal transduction pathway to three G1-phase Cdc28 kinase complexes. *Mol. Cell. Biol.* **13**, 5659-5669 (1993).
53. Heinrich, M., Köhler, T. & Mösch, H. Role of Cdc42-Cla4 interaction in the pheromone response of *Saccharomyces cerevisiae*. *Eukaryotic cell* **6**, 317-327 (2007).
54. Strathern, J., Hicks, J. & Herskowitz, I. Control of cell type in yeast by the mating type locus: the $\alpha 1$ - $\alpha 2$ hypothesis. *J. Mol. Biol.* **147**, 357-372 (1981).

55. Alifano, P. *et al.* Histidine biosynthetic pathway and genes: structure, regulation, and evolution. *Microbiol. Rev.* **60**, 44 (1996).
56. Fink, G. Gene-enzyme relations in histidine biosynthesis in yeast. *Science* **146**, 525-527 (1964).
57. McGrath, J. P. & Varshavsky, A. The yeast STE6 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. *Nature* **340**, 400-404 (1989).
58. Sherman, F., Fink, G. & Lawrence, C. in *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory Press, New York, 1974).
59. Reid, B. J. & Hartwell, L. H. Regulation of mating in the cell cycle of *Saccharomyces cerevisiae*. *J. Cell Biol.* **75**, 355-365 (1977).
60. Gonçalves-Sá, J. & Murray, A. Asymmetry in sexual pheromones is not required for ascomycete mating. *Current Biology* **21**, 1337-1346 (2011).
61. Lee, P. J., Helman, N. C., Lim, W. A. & Hung, P. J. A microfluidic system for dynamic yeast cell imaging. *BioTechniques* **44**, 91-95 (2008).
62. Sprague, G. F., Jr. Assay of yeast mating reaction. *Methods Enzymol.* **194**, 77-93 (1991).
63. Collart, M. A. & Oliviero, S. Preparation of yeast RNA. *Current protocols in molecular biology*, 13.12. 1-13.12. 5 (2001).
64. Trapnell, C., Pachter, L. & Salzberg, S. L. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105-1111 (2009).
65. Trapnell, C. *et al.* Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **28**, 511-515 (2010).

CHAPTER 3

Polarized secretion and restricted diffusion of cell wall remodeling enzymes induces cell wall dissolution in pheromone-stimulated yeast cells

ABSTRACT

Cell fusion is an important event in the life cycle of many organisms. In humans it is necessary for muscle cell formation and the fertilization of an egg by sperm. A useful model system for studying cell fusion is cell fusion of the budding yeast, *Saccharomyces cerevisiae*, which occurs when two haploid cells of opposite mating types signal through reciprocal pheromones and pheromone receptors, grow towards each other, and fuse to form a single diploid cell. Since yeast cells have both a cell wall and a plasma membrane, this requires both cells to first dissolve their cell walls at the point of contact and is a dangerous task because the osmotic pressure differential between the cytoplasm and extracellular environment is high enough that when the plasma membrane is unprotected, water rushes into the cell causing it to lyse. If a cell dissolves its cell wall at an inappropriate time or location, the cell will die. We hypothesize that cell wall dissolution during budding yeast mating is the result of a contact-driven increase in the local concentration of cell wall remodeling enzymes. When a cell is unattached, the secreted cell wall remodeling enzymes are able to diffuse directly out of the cell wall. However, when two cells are attached, the cell wall remodeling enzymes must diffuse laterally in order to escape the fusion zone, increasing the time spent in the cell wall and, thus, the concentration of cell wall remodeling enzymes leading to cell wall dissolution. To study this we tightly apposed pheromone-stimulated cells to non-permeable surfaces and observed cell lysis using microscopy. We found that pheromone-induced cell lysis increases in frequency with increasing pheromone concentration and decreases in frequency when putative glucanases previously shown to be involved in mating or genes known to be necessary for efficient cell wall fusion are deleted giving strong support to our hypothesis.

INTRODUCTION

Cell fusion is an important event in the life cycle of many organisms. In mammals, it is necessary for fertilization of an egg by sperm and formation of myoblasts, osteoclasts, giant cells, and placental cells¹⁻⁴. It is also important in the development of *Caenorhabditis elegans* and *Drosophila melanogaster*, as well as in the life cycle of many algae, such as *Chlamydomonas reinhardtii* and fungi, such as *Neurospora crassa*⁵⁻⁸. Perhaps the simplest and most well studied form of cell fusion is the mating of the budding yeast, *Saccharomyces cerevisiae*⁹.

Budding yeast can exist in both a diploid and haploid state. In either state, cells can replicate asexually by budding, producing daughters that are genetically identical to their mothers. Haploid cells can be one of two mating types: *MATa* or *MAT α* . When two haploid cells of opposite mating types come in contact with one another, they are capable of fusing to form a single diploid *MATa/MAT α* cell¹⁰.

Intercellular signaling between budding yeast cells is important for efficient mating. Signaling is achieved through G-protein coupled receptors on the plasma membrane of each cell, which bind pheromones produced by cells of the opposite mating type¹¹. *MAT α* cells produce α -factor and express the **a**-factor receptor, Ste3, on their surface¹²⁻¹⁴. *MATa* cells produce **a**-factor and express the α -factor receptor, Ste2, on their surface^{15, 16}. When *MATa* cells are grown in cultures containing only other *MATa* cells, **a**-factor and Ste2 are expressed at low levels^{17, 18}. However, when Ste2 binds α -factor, a mitogen-activated protein (MAP) kinase cascade, which is conserved in other ascomycete fungi, is started, stimulating the cell to produce higher levels of its own pheromone, **a**-factor, and the receptor for α -factor, Ste2, as well as upregulating other proteins involved with mating^{11, 19-21}. Pheromone stimulation arrests *MATa* cells in G1, polarizes

them in the direction of highest pheromone concentration, and induces polarized cell growth to form a mating projection known as a shmoo^{19, 22-24}. *MAT α* cells produce a similar, coordinated response using the reciprocal G-protein coupled receptor (Ste3) and mating pheromone^{12-14, 18-20, 22, 23}.

After *MAT α* and *MAT α* cells have successfully communicated and grown towards each other, they must fuse¹⁰. The two cells initially bind to each other at their shmoo tips using mating agglutinins²⁵⁻²⁷, but their plasma membranes are still separated by approximately 100nm thick cell walls²⁸. Before the mating partners can fuse, the cell wall that lies between the two membranes must be dissolved and the boundaries of the remaining cell walls, which surround the site of cell fusion, must fuse to form a single, continuous structure that will surround the newly formed zygote⁹. The osmotic pressure differential between the cytoplasm and the extracellular environment makes this spatially regulated cell wall dissolution and fusion a dangerous task^{29, 30}. If the cell wall is opened at the wrong time or in an inappropriate location, exposing the plasma membrane directly to the environment, there will be no elastic force to resist the turgor pressure of the cell, water will rush into the cell from the extracellular environment, and the cell will lyse^{29, 30}.

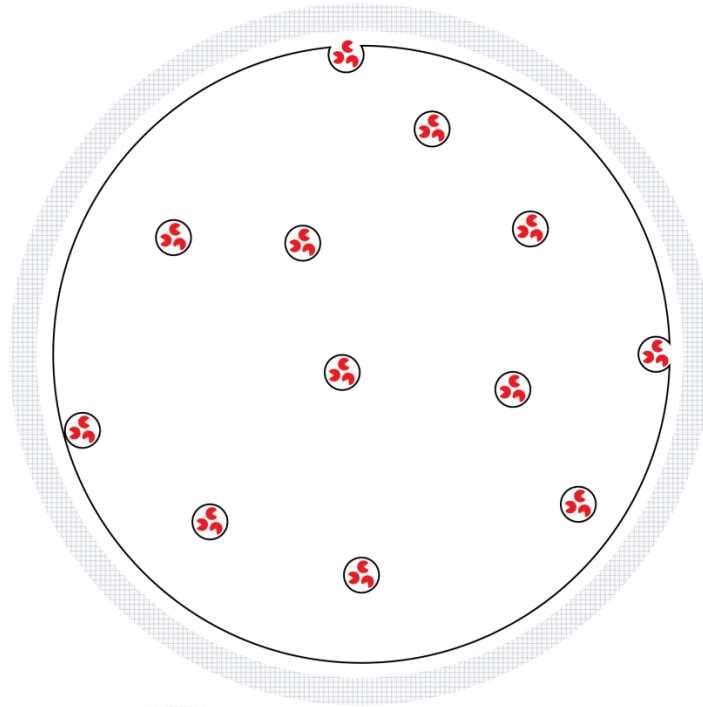
Various studies have been done on the molecular basis for cell wall dissolution. In 1996, Brizzio *et al.* showed that high pheromone concentrations are required for efficient fusion and hypothesized that numerous vesicles found at the shmoo tip might contain cell wall remodeling enzymes³¹. Later, Cappellaro *et al.* found several proteins with homology to known cell wall glucanases, including *SCW4* and *SCW10*, whose deletion makes mating less efficient³². The genes for two putative glucanases *SCW10* and *SCW11*, another gene found by Cappellaro *et al.*³², were also found to have binding sites for Ste12, the transcription factor that regulates genes in

response to pheromone stimulation³³, in their promoters²⁰. The vesicles found in the shmoo tip are hypothesized³⁴ to be transported to the cell fusion zone by a complex containing Rvs161, an amphiphysin-like protein that binds curved membranes^{35, 36} and is involved in cell fusion³⁷, and Fus2^{38, 39}, another protein known to be involved in cell fusion⁴⁰. Once Fus2 and its hypothesized vesicle cargo has reached the plasma membrane, it is anchored there by Fus1³⁴, a membrane spanning protein⁴¹ involved with cell fusion⁴⁰ that interacts with the polarisome⁴², a protein complex associated with polarized actin polymerization,⁴³ presumably ensuring tight clustering of the secretory vesicles. However, prior to cell fusion, the cell wall must be dissolved, and none of the methods that have been proposed to regulate cell wall dissolution limit this remodeling to the site of fusion with a polarized partner.

The problem of remodeling the cell wall is not unique to mating. Even when cells are growing isotropically, there must be a balance between cell wall synthesis and destruction to allow the continual increase in cell diameter and volume^{44, 45}. Cell wall-modifying enzymes can either be attached to the plasma membrane or cell wall or be secreted and free to diffuse through the cell wall, and these enzymes can be involved in synthesizing and cross-linking the polysaccharides that make up much of the cell wall or in cleaving these bonds⁴⁶. These two processes must be carefully balanced: an excess of synthesis over degradation will lead to an increased cell wall thickness and eventually slow growth, whereas an excess of degradation will thin the cell wall to the point that it is unable to resist the osmotic pressure inside the cell⁴⁷.

In an isotropically growing cell, synthetic and remodeling enzymes are secreted uniformly around the cell, whereas the polarized growth that accompanies both budding and shmooing requires similarly polarized secretion of these enzymes⁴⁵ (Figure 3.1). Due to this polarized secretion during pheromone stimulation, we hypothesize that cell wall remodeling

A.



B.

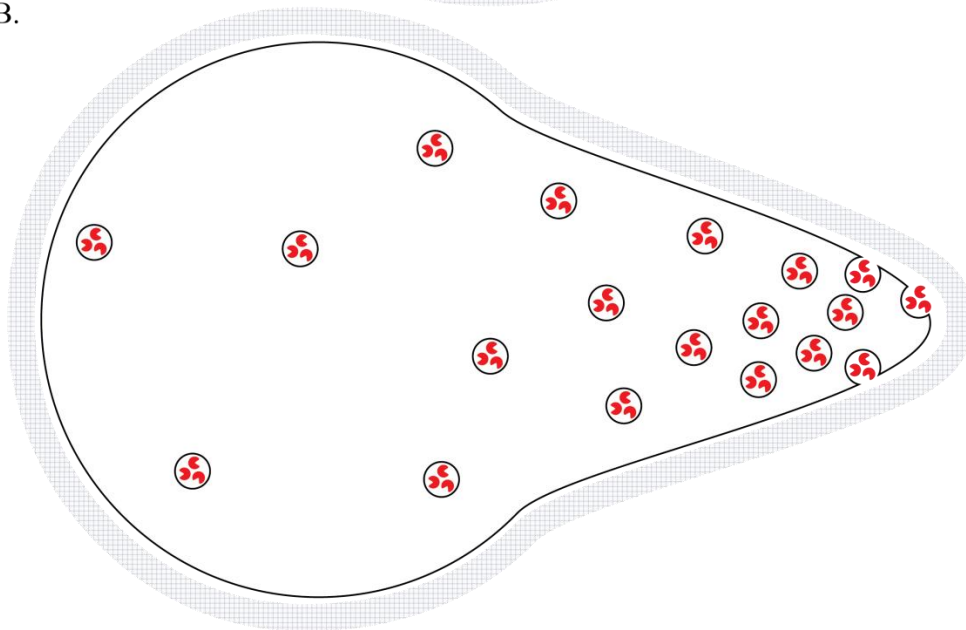
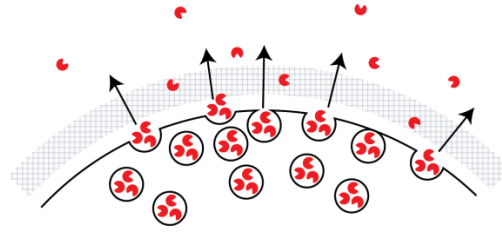


Figure 3.1. Secretion in isotropically growing and polarized cells. **A.** Isotropically growing cells must increase the size of their cell walls equally in all directions to grow larger while maintaining a spherical shape, so cell wall remodeling enzymes are secreted equally in all directions. **B.** Polarized cells grow in a particular direction, so they must polarize secretion of cell wall remodeling enzymes in order to preferentially expand their cell walls in the direction of polarization.

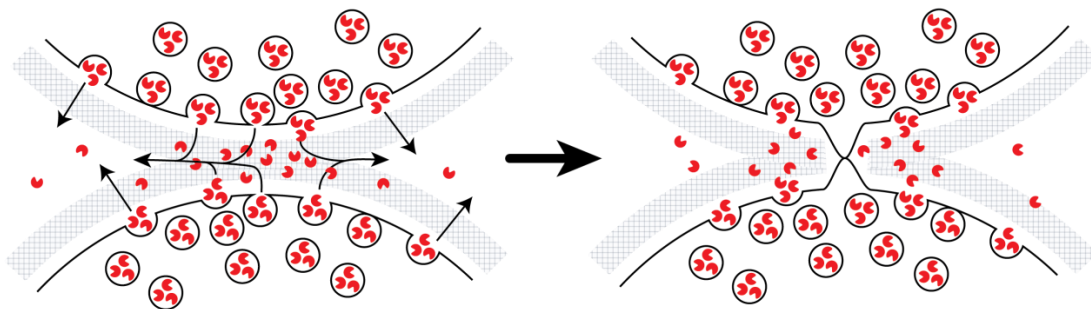
enzymes, such as Scw4, Scw10, and Scw11, are preferentially released at the shmoo tip, which causes cell wall weakening and allows for continual expansion of the shmoo in the direction of highest pheromone concentration. As a shmoo approaches a suitable partner, the concentration of pheromone increases, tightening the polarization, and increasing the concentration of cell wall remodeling enzymes in the part of the cell wall that has polarized towards the location of highest pheromone stimulation⁴⁸⁻⁵². If the remodeling enzymes are diffusible, the maximum concentration they can reach in a shmoo that has not bound to a partner is limited: even though the secretion rate of cell wall remodeling enzymes is high, the enzymes are able to diffuse through the cell wall, keeping their concentration in the cell wall low enough to balance the need for rapid growth with cell wall rupturing (Figure 3.2A). However, when two shmoo tips are attached to each other via mating agglutinins, it takes longer for cell wall remodeling enzymes to diffuse out of the fusion zone because they must now travel laterally through the cell wall in order to escape, thus increasing the local concentration of the remodeling enzymes and causing the cell walls of the shmoo tips to dissolve specifically at the point of attachment (Figure 3.2B).

Previous studies have reported cell death after exposure to high pheromone concentrations^{53, 54}. Zhang *et al.* proposed that this death was due to inappropriate activation of cell fusion machinery, resulting in cell wall dissolution and eventual cell lysis⁵⁴. Although, Zhang *et al.* observed that this lysis can be regulated by increasing cell wall integrity and deleting certain proteins involved in cell fusion, they did not hypothesize a mechanism through which this process is regulated⁵⁴. We propose that the reported death is due to tight apposition of cells to non-permeable surfaces, so in this study we investigated the hypothesis that the cell wall dissolution required for mating is the result of a contact-driven increase in the local concentration of cell wall remodeling enzymes. We tightly apposed pheromone-treated cells to a non-

A.



B.



C.

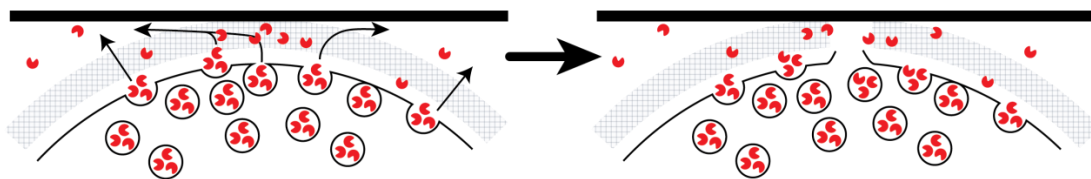


Figure 3.2. Model: Pheromone-stimulated cell wall dissolution is the result of a contact-driven increase in the local concentration of cell wall remodeling enzymes. **A.** When pheromone stimulated cells are unattached, the cell wall remodeling enzymes secreted from the shmoo tip are able to exit the cell wall along the shortest possible path, i.e. the line perpendicular to the tangent of the curve of the cell. This causes cell wall weakening and allows for continual expansion of the shmoo in the direction of highest pheromone concentration but does not breach the cell wall. **B.** When two pheromone-stimulated cells are attached by mating agglutinins, the cell wall remodeling enzymes secreted into the fusion zone must now travel a much further distance to exit the cell wall, i.e., laterally along the tangent of the curve of the cell, increasing the local concentration of cell wall remodeling enzymes in the fusion zone. The action of cell wall remodeling enzymes dissolves the two cell walls specifically at the point of contact while simultaneously interlocking them, allowing the plasma membranes of the two cells to come in

(Figure 3.2 continued) contact with one another and fuse. **C.** To mimic the attachment of two cells by mating agglutinins, we tightly appose cells to non-permeable membranes, forcing cell wall remodeling enzymes to exit the cell wall laterally along the tangent of the curve of the cell and increasing the concentration of cell wall remodeling enzymes at the point of attachment to the non-permeable surface. This causes a hole to form in the cell wall of the cell, exposing the plasma membrane to the extra-cellular environment and causing the cell to lyse due to the osmotic pressure differential.

permeable surface, mimicking the effect of cell-cell attachment during mating (Figure 3.2C). This manipulation induces cell wall lysis whose frequency is reduced by the deletion of putative glucanases and genes known to be involved in cell wall fusion during mating as well as decreasing the osmotic pressure difference between the cell and its environment and increased by increasing the osmotic pressure difference between the cell and its environment.

RESULTS

Cells lyse when attached to a non-permeable surface

We set out to investigate the hypothesis that cell wall remodeling enzymes are constitutively secreted into the extracellular environment during pheromone stimulation and only dissolve the cell wall when their diffusion is restricted by collision with a barrier. We began by looking at the response of pheromone-stimulated cells grown in bulk culture. To do this, *MATa* cells that were either deleted or wild-type for the α -factor protease, Bar1⁵⁵ were grown to log phase and then incubated in media with α -factor. Because *bar1Δ* cells are significantly more sensitive to α -factor than *BARI*⁺ cells⁵⁶, we stimulated *bar1Δ* cells with 50nM α -factor and *BARI*⁺ cells with 10μM α -factor, observed all cells after 5 hours of pheromone stimulation, and found that roughly 10% of the cells in each of the bulk cultures appear to lyse (Figure 3.3). We investigated the lysis of the *bar1Δ* and *BARI*⁺ cells and noticed that most of the lysed cells appeared to be in cell clumps as opposed to floating freely as single cells (Figure 3.4). Although both mating agglutinins are required for pheromone-induced agglutination to occur⁵⁷, cultures containing only a single mating type can still form clumps due to incomplete separation after budding⁵⁸. Therefore, we theorized that the cells lyse because they are attached to another cell or potentially have come in contact with the non-permeable surface of the culture tube, causing a

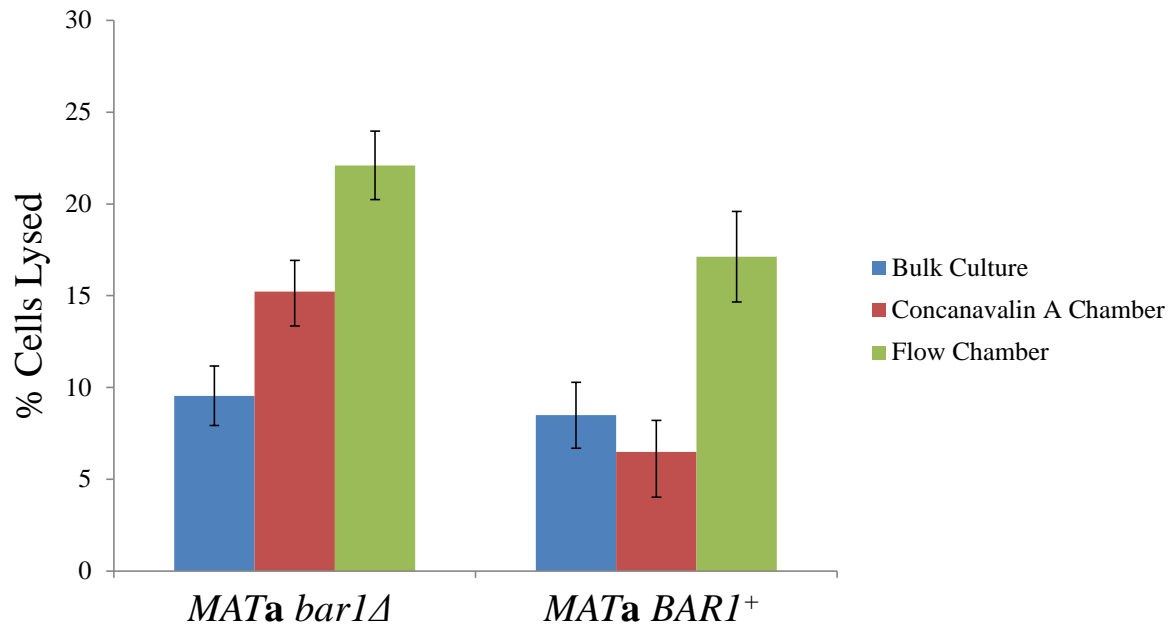
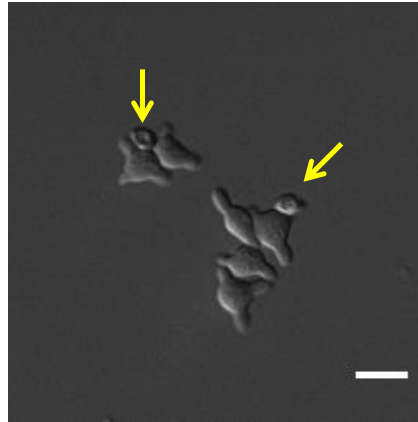


Figure 3.3. Pheromone-stimulated cell lysis is dependent on attachment to a non-permeable surface. *MATa* cells that were either deleted or wild-type for the α -factor protease gene, *BARI*, were pheromone stimulated in rich media in three different environments for five hours, imaged, and then the number of lysed and live cells was counted. *BARI⁺* cells were grown in 10 μ M α -factor. *bar1Δ* cells were grown in 50nM α -factor. Bulk culture refers to cells that were incubated in test tubes in liquid media. Concanavalin A chamber refers to a chamber many times the diameter of a single yeast cell in which cells were attached to a glass coverslip using the lectin, concanavalin A. The chamber was filled with rich media containing α -factor using capillary action and the cells were observed using microscopy. Flow chamber refers to a microfluidics chamber whose floor and ceiling are separated by the height of a single yeast cell through which new media is constantly perfused. The floor of the chamber is glass, so the cells can be observed using microscopy.

A.



B.

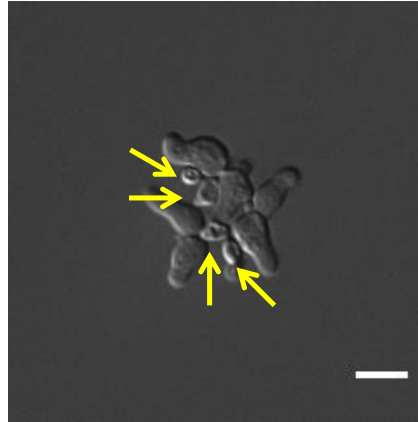


Figure 3.4. Pheromone-stimulated cell lysis in bulk culture occurs mainly in cell clumps.
A. *MATa bar1Δ* cells were grown in rich media containing 50nM α -factor in a culture tube in liquid media for 5 hours and then imaged using 20x magnification. Yellow arrows point to cells that have lysed. The scale bar indicates 10 μ m. **B.** *MATa BARI⁺* cells were grown in rich media containing 10 μ M α -factor in a culture tube in liquid media for 5 hours and then imaged using 20x magnification. Yellow arrows point to cells that have lysed. The scale bar indicates 10 μ m.

high concentration of cell wall remodeling enzymes to build up in the part of the cell wall that is tightly apposed to the non-permeable surface.

Bulk culture limits our ability to observe individual cells and accurately control their environment. We used two techniques to overcome these limitations and study the effect of attaching pheromone-stimulated cells to a non-permeable surface. To mimic the attachment of two cell walls via mating agglutinins, we first attached cells to the non-permeable surface of a glass coverslip using the lectin, concanavalin A, which binds to carbohydrates in the cell wall⁵⁹. In order to image the yeast cells for an extended period of time, we created a chamber that was several hundred times the diameter of a yeast cell. The chamber was filled with medium containing α -factor using capillary action and then sealed and observed.

We found that *MATa bar1 Δ* cells attached covalently to a non-permeable surface were 1.6 times more likely to lyse than those in bulk culture, indicating that attachment to a non-permeable surface increases the rate of cell lysis ($p < 0.02$) (Figure 3.3). We did not see a significant change in the rate of lysis of *MATa BARI⁺* cells (Figure 3.3). This may be because as the cells incubated in the relatively small volume of media in the chamber, the Bar1 secreted by the wild-type cells began to cleave the α -factor present, decreasing the α -factor concentration and, thus, the level of pheromone-stimulation the cells in the chamber were experiencing, causing less polarized shmoos^{55, 56}. Indeed, not all of the *MATa BARI⁺* cells attached to the concanavalin-A coated coverslip were pheromone-stimulated (Figure 3.5A). Also, because the pheromone was uniformly present in the media, the cells polarized randomly, which meant that many of the cells, both *BARI⁺* and *bar1 Δ* , polarized away from the non-permeable surface (Figure 3.5B-C).

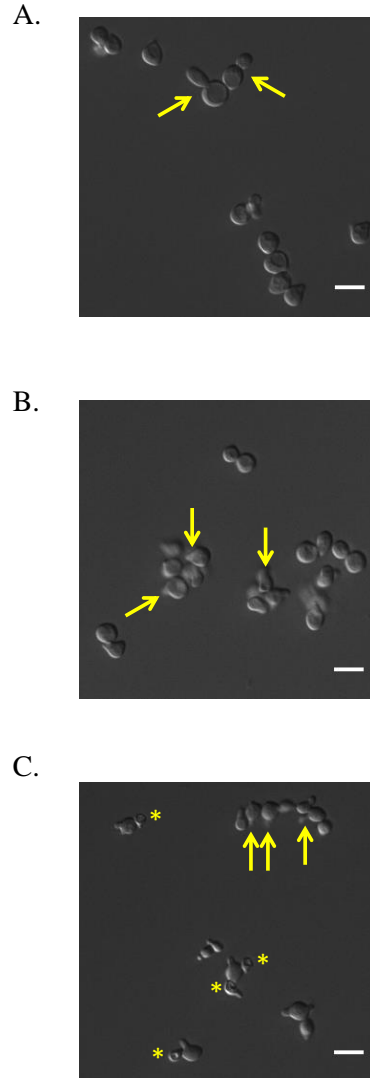


Figure 3.5. Bar1 produced by wild-type *MATa* cells reduces the α -factor concentration and cells with essentially infinite space to expand can polarize away from the non-permeable surface. *MATa* *BAR1*⁺ cells were attached to a glass coverslip using concanavalin A and incubated in a chamber with a finite amount of rich media with 10 μ M α -factor but infinite space to expand for 5 hours. Cells were imaged using 20x magnification. **A.** Yellow arrows point to budding cells. The scale bar indicates 10 μ m. **B.** Yellow arrows point to cells that are polarizing away from the non-permeable surface of the glass coverslip. The scale bar indicates 10 μ m. **C.** *MATa* *bar1* Δ cells were attached to a glass coverslip using concanavalin A and incubated in a chamber with a finite amount of rich media with 50nM α -factor but infinite space to expand for 5 hours. Cells were imaged using 20x magnification. Yellow arrows point to cells that are polarizing away from the non-permeable surface of the glass coverslip. Yellow (*) indicate cells that have lysed. The scale bar indicates 10 μ m.

To at least partially address these problems we used a second technique to mimic the attachment of two cell walls via mating agglutinins. We trapped cells in a microfluidics chamber whose floor and ceiling are separated by the height of a single yeast cell through which new media is constantly perfused. Cells are loaded into this device and then trapped between a silicone ceiling and a glass floor, which means that not only are the cells tightly apposed to two non-permeable surfaces, but they are also more likely to polarize towards an impermeable surface. Using an inverted microscope, it is possible to image cells over time through the glass floor as media is perfused through the chamber. Once again we imaged both *MATa BARI⁺* and *MATa bar1Δ* cells in α -factor for 5 hours. In the flow chamber, the rate of lysis of the *MATa BARI⁺* cells was more than twice as high as the rate of lysis in bulk cultures or when attached to concanavalin A-coated coverslips ($p < 0.02$) (Figure 3.3). Similarly, the rate of lysis of the *MATa bar1Δ* cells in the flow chamber was more than twice as high as in bulk culture and 1.5 times the rate of lysis when attached to concanavalin A-coated coverslips, suggesting that a larger area of attachment to a non-permeable surface causes increased cell lysis ($p < 0.02$) (Figure 3.3 and Movie A3).

We asked whether the lysis rate is dependent on the concentration of α -factor in the media, by perfusing different concentrations of α -factor through the flow chamber and determining the percentage of cells that lyse in 5 hours. We decreased the concentration of α -factor the *MATa bar1Δ* cells were exposed to and compared the percentage of lysed cells to that seen in 50nM α -factor. As expected, decreasing the α -factor concentration decreased the percentage of cells that lysed in the flow chamber. In 5nM α -factor, less than 1% of cells lysed in the flow chamber, and as the concentration of α -factor was increased, causing cells to form

more tightly polarized shooms, the percentage of lysed cells increased up to 22% in 50nM α -factor ($p < 0.02$) (Figure 3.6).

The flow chamber traps cells by wedging them into a space minutely smaller than a single cell in height. When cells are arrested, such as by pheromone stimulation, the cells increase in size as they continue to grow without dividing⁶⁰. Because of this, it is possible that the increased frequency of cell lysis in the flow chamber as compared to bulk culture and when cells are attached to concanavalin A-coated coverslips is not due to an increased amount of cell surface apposed to a non-permeable surface but rather because the physical strain put on the cell wall is too high, particularly when media is perfused through the chamber at 14 kPa (2psi). We therefore used a different method to arrest the cell cycle without interfering with cell growth. Like pheromone treatment, treating cells with benomyl causes cells to become larger without dividing, but unlike pheromone-arrest, benomyl-arrested cells are unpolarized and arrest in mitosis instead of G1⁶¹ (Figure 3.7A). If cells in the flow chamber lysed because they were squashed, a similar percentage of benomyl-arrested and α -factor arrested cells should lyse in the flow chamber. Alternatively, if lysis reflects polarized secretion of cell wall remodeling enzymes towards an impermeable surface, the rate of lysis should be significantly lower in benomyl-arrested cells than in pheromone-arrested cells. Although it is possible to find the occasional lysed benomyl-arrested cell, less than 0.5% of these cells lyse, indicating that lysis in the flow chamber is specific to pheromone-arrest as opposed to arrest in general (Figure 3.7B).

1M sorbitol can mitigate the lysis of cells attached to a non-permeable surface

Yeast cells require cell walls at least in part due to osmotic pressure. Since the osmolarity of the cytoplasm is higher than the typical extracellular environment, without the rigidity of a cell wall, water would rush into the cell and cause it to lyse⁴⁵. One interpretation of

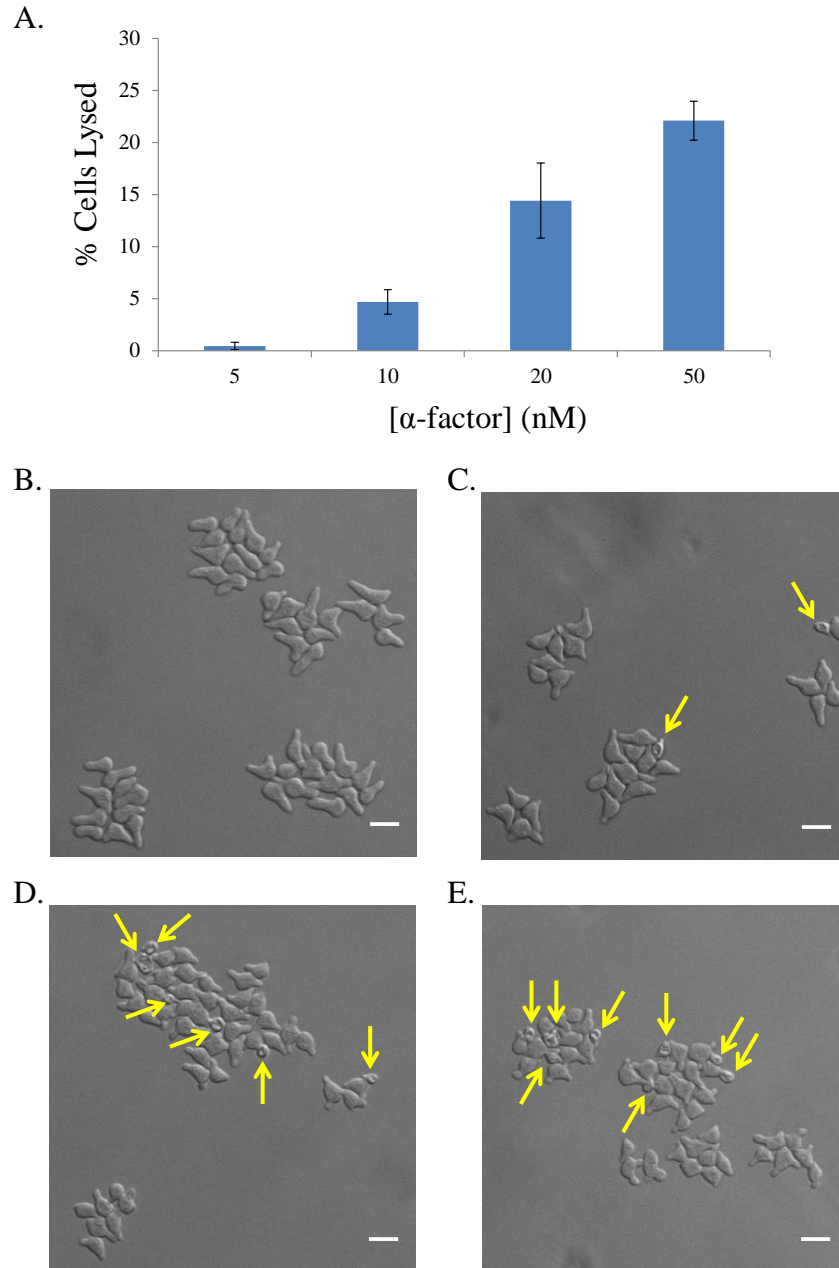
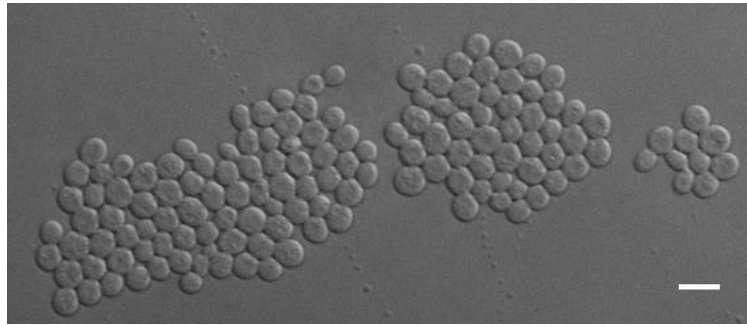


Figure 3.6. Increasing α -factor concentration increases the tightness of polarization and lysis frequency of *MATa bar1Δ* cells. *MATa bar1Δ* cells were grown in a flow chamber for 5 hours perfused with rich media with 5nM, 10nM, 20nM, or 50nM α -factor and imaged using 20x magnification. **A.** Percentage of cells that lysed after 5 hours. **B.** Cells grown in 5nM α -factor. Yellow arrows indicate cells that have lysed. The scale bar indicates 10 μ m. **C.** Cells grown in 10nM α -factor. Yellow arrows indicate cells that have lysed. The scale bar indicates 10 μ m. **D.** Cells grown in 20nM α -factor. Yellow arrows indicate cells that have lysed. The scale bar indicates 10 μ m. **E.** Cells grown in 50nM α -factor. Yellow arrows indicate cells that have lysed. The scale bar indicates 10 μ m.

A.



B.

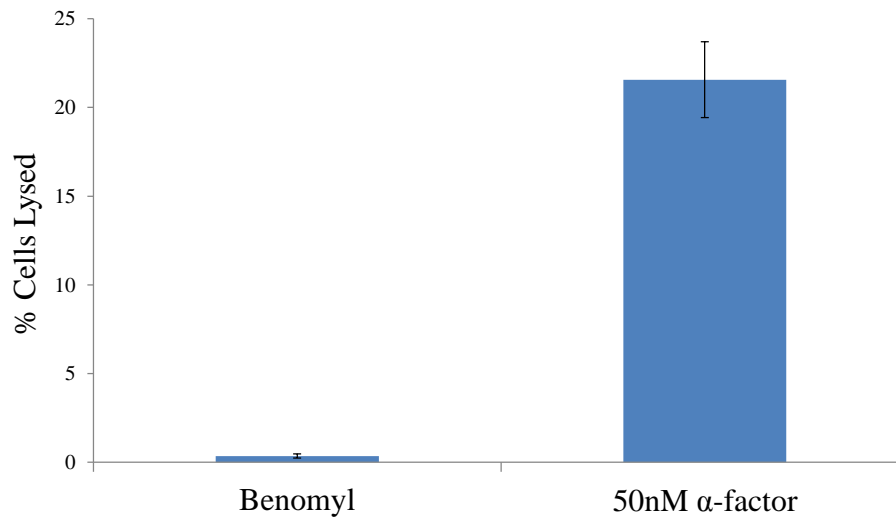


Figure 3.7. Benomyl arrested cells rarely lyse in the flow chamber. Cells were grown in a flow chamber for 5 hours perfused with rich media with 0.1mM benomyl or rich media with 50nM α -factor and imaged using 20x magnification. **A.** Benomyl-arrested cells in the flow chamber. The scale bar indicates 10 μ m. **B.** Percentage of cells that lysed after 5 hours ($p < 10^{-6}$).

the death of pheromone-treated cells pressed against an impermeable surface is that they are digesting part of their cell walls leading to membrane expansion through a hole in the cell wall and eventual lysis. If this interpretation is correct, it should be possible to affect the rate of lysis by manipulating the osmotic pressure differential between the cell and the medium⁶².

Although the deaths seen in the flow chamber appear to be caused by osmotic lysis, it is possible they are due to another cause. We did two experiments to determine whether the pheromone-induced deaths are due to osmotic lysis: increasing the osmotic pressure differential between the cytoplasm and the extracellular environment and decreasing the osmotic pressure differential between the cytoplasm and the extracellular environment. To change the osmolarity of the media we added 1M sorbitol so that the osmolarity of the extracellular environment more closely mimicked the osmolarity of the cytoplasm. Deaths due to osmotic lysis should be mitigated by the presence of 1M sorbitol. Because *MATa bar1Δ* cells exposed to 50nM α -factor in the flow chamber have the most severe lysis phenotype, we chose these conditions to assay for whether osmoprotection mitigates cell death.

We first tested the effect of increasing the osmotic pressure differential between the cytoplasm and the extracellular environment. Cells were exposed to rich media with 50nM α -factor and 1M sorbitol for 5 hours. When in hyperosmotic conditions, cells adapt to the osmotic stress by synthesizing glycerol, which can take place in a matter of minutes⁶³⁻⁶⁶. Because of this we were unsurprised to find that approximately one fifth of the cells lysed while incubated in the 1M sorbitol. However, if our hypothesis is correct, it seems reasonable to assume that many of the live cells also have holes in their cell walls and that the presence of the 1M sorbitol is protecting these cells from death. Thus, if we replace the sorbitol-containing media with media lacking sorbitol, we would expect to see rapid cell death as water diffuses into these cells and

causes them to burst. To test this, we washed the media containing 1M sorbitol and α -factor out of the flow chamber after the cells had been exposed for 5 hours and replaced it with media containing only 50nM α -factor. As expected, immediately following the sorbitol washout, the number of lysed cells in the flow chamber more than doubled, supporting the idea that the cells in the chamber are lysing due to a breach in their cell walls as opposed to death for some other reason (Figure 3.8A-C and Movie A4).

To test the effect of decreasing the osmotic pressure differential between the cells and the extracellular environment, we exposed cells to 50nM α -factor in the absence of 1M sorbitol for 80 minutes, at which point cells are just beginning to lyse (Movie A3). We determined the percentage of cells lysed at this point and then perfused the chamber with media containing 1M sorbitol and α -factor and observed the percentage of lysed cells 60 minutes after the change of media. Since the sorbitol is washed in after the cells have begun to shmoo, the cells will have less time to induce the hyperosmotic response, and if the cell death is due to osmotic lysis, we should observe fewer cell deaths when 1M sorbitol is present in the media. When we observe the fold change in cell death between 80 minutes and 140 minutes after α -factor addition in the absence of 1M sorbitol, there is an 8.9-fold increase in the percentage of dead cells (Figure 3.8D). However, when 1M sorbitol is added to the media 80 minutes after α -factor addition, there is only a 1.4-fold increase in the percentage of dead cells between 80 and 140 minutes after α -factor addition, further indicating that the observed pheromone-induced cell death is due to cell wall dissolution (Figure 3.8D).

Cells lacking cell wall remodeling enzymes lyse less frequently than wild-type cells

Several cell wall remodeling enzymes have been implicated in mating, including three putative glucanases, Scw4, Scw10, and Scw11^{ref. 32}. If our hypothesis is correct and if these

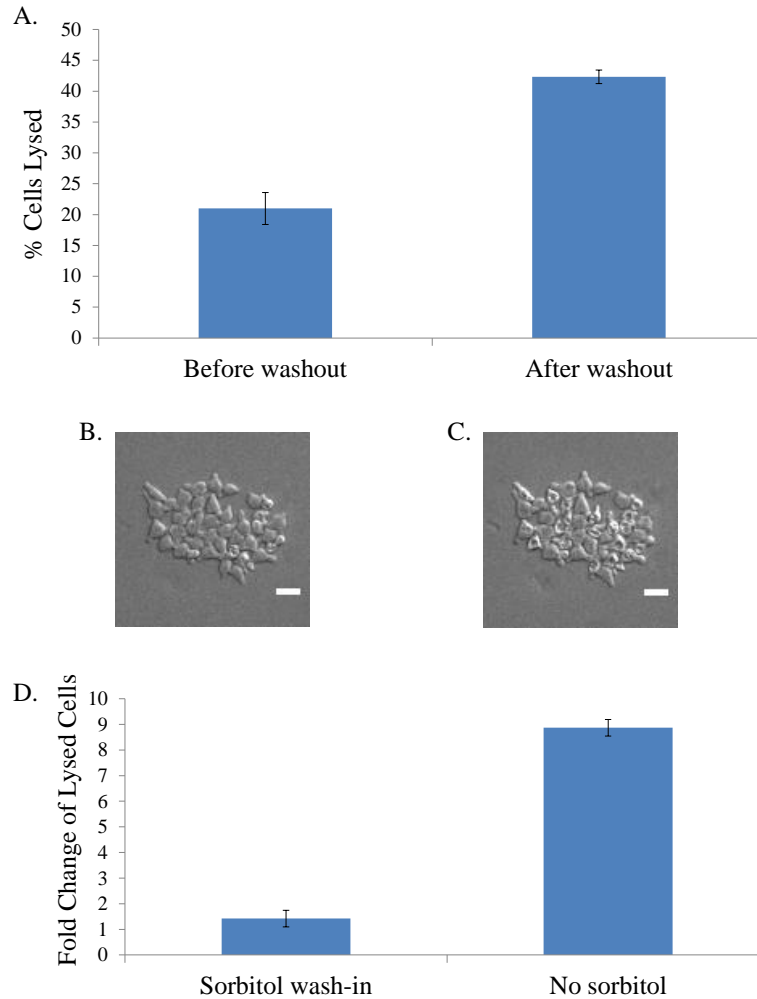


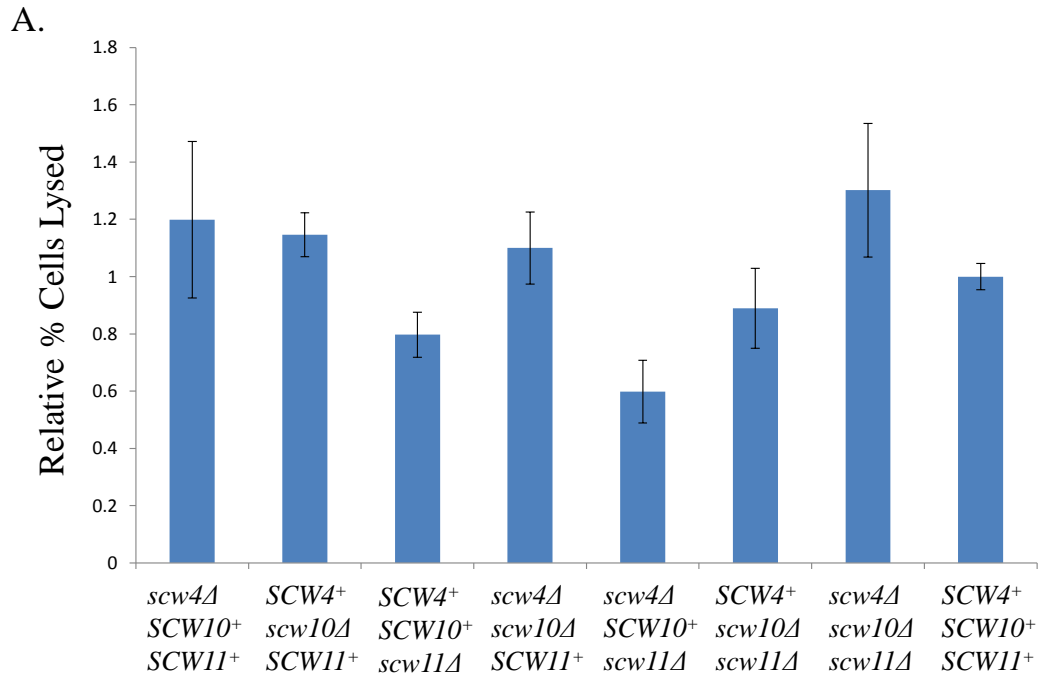
Figure 3.8. 1M sorbitol protects cells from pheromone-induced cell lysis. **A.** Cells were grown in a flow chamber for 7 hours. For the first 5 hours the chamber was perfused with rich media with 1M sorbitol and 50nM α -factor. At the end of 5 hours, the media in the chamber was replaced with rich media with 50nM α -factor and no sorbitol. Cells were imaged using 20x magnification, and the percentage of cells lysed 10 minutes before and 10 minutes after the 1M sorbitol was washed out was determined ($p=2 \times 10^{-4}$). **B.** Cells imaged after 4h50m in rich media with 1M sorbitol and 50nM α -factor (10 minutes prior to the washout of the sorbitol). The scale bar indicates 10 μ m. **C.** Cells imaged 10 minutes after the media was replaced with rich media with 50nM α -factor and no sorbitol. The scale bar indicates 10 μ m. **D.** Cells were grown in the flow chamber for 80 minutes perfused with rich media with 50nM α -factor. After 80 minutes, the chamber was perfused with rich media with 1M sorbitol and 50nM α -factor. Cells were imaged using 20x magnification, and the fold change in cells lysed immediately prior to and 60 minutes after the sorbitol wash-in was determined. In control chambers, no sorbitol was added to the media, and the fold change in cells lysed 80 minutes and 140 minutes after the addition of α -factor was determined ($p=9 \times 10^{-5}$).

enzymes constitute a majority of the cell wall remodeling enzymes required for cell wall fusion, deleting these genes should reduce the frequency of pheromone-induced cell death in the flow chamber. To test this prediction, we grew *MATa bar1Δ* cells with *SCW4*, *SCW10*, and *SCW11* deleted alone and in combination and monitored their lysis in a flow chamber where they were exposed to 50nM α -factor for 5 hours.

Deleting *SCW4* and *SCW10* individually did not decrease the number of cell lysis events in the flow chamber (Figure 3.9A). However, deleting *SCW11* caused a 20% reduction in cell lysis compared to *MATa bar1Δ* cells and additionally deleting *SCW4* in this strain caused cell lysis to reduce by 40% compared to *MATa bar1Δ* cells ($p < 10^{-3}$) (Figure 3.9A). Cells that had *SCW10* deleted in combination with either of the other two proteins did not have a reduced cell lysis frequency (Figure 3.9A). However, all three of these proteins have additional functions in vegetative cell wall maintenance^{67, 68}, and cells deleted for *SCW10* in combination with other cell wall proteins have abnormal cell morphology, slow growth, and increased sensitivity to the cell wall-weakening agent calcoflour white³². Therefore, it is possible that the cell walls of these cells are more brittle than wildtype causing these cells to lyse more frequently in the constraints of the flow chamber. To test this, we observed *MATa bar1Δ scw4Δ scw10Δ scw11Δ* cells growing vegetatively in the flow chamber in rich media without α -factor and saw that these cells die in the flow chamber even when they are not large and arrested (Figure 3.9B). However, the fact that deleting *SCW11* alone and in combination with *SCW4* has a significant effect on the rate of cell lysis indicates that these putative glucanases are involved in the observed cell lysis.

FUS1 and FUS2 are required for cell wall breakdown

Although *Scw4* and *Scw11* have been implicated in mating, they are also involved in other aspects of yeast cell wall maintenance, such as breakdown of the cell wall during daughter



B.

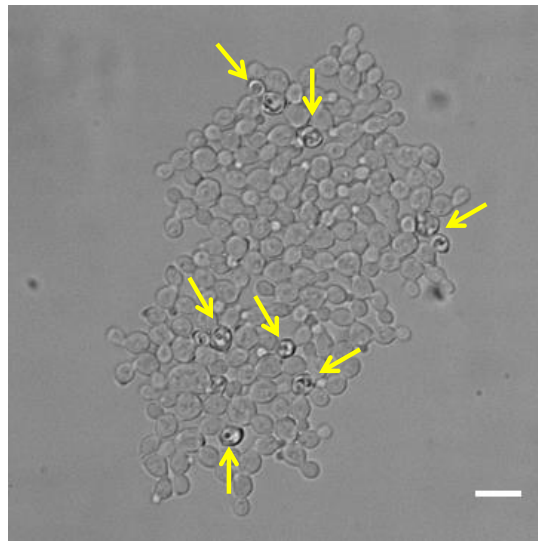


Figure 3.9. The deletion of the putative glucanases Scw4 and Scw11 mitigates pheromone-induced cell lysis. **A.** Percentage of cell lysis in *MATa bar1Δ* cells lacking different combinations of putative glucanases implicated in mating relative to *MATa bar1Δ* cells grown in a flow chamber for 5 hours perfused with rich media with 50nM α -factor. **B.** *MATa bar1Δ scw4Δ scw10Δ scw11Δ* cells grown in a flow chamber for 5 hours perfused with rich media and imaged using 20x magnification. Yellow arrows point to dead cells. The scale bar indicates 10 μ m.

separation after budding^{67, 68}. Because of this, we could not be sure if the decrease in cell lysis events in the flow chamber in cells lacking these genes compared to wildtype is specific for pheromone stimulation or because of unrelated differences in the cell wall structure of these strains. The genes *FUS1* and *FUS2* have both been shown to be involved in cell fusion and specifically cell wall fusion during mating but do not have a known function in vegetative cell wall maintenance⁴⁰. When *FUS1*, *FUS2*, or, both *FUS1* and *FUS2* are deleted in both parents, prezygotes are formed, but cells are unable to dissolve their cell walls to facilitate cell fusion⁴⁰. Also, in *fus1* and *fus1fus2* mutants, the tightly polarized vesicles seen in the fusion zone of wild-type prezygotes hypothesized to contain cell wall remodeling enzymes are largely absent and more widely dispersed than in wild-type cells⁶⁹. We hypothesize that if cell lysis in the flow chamber is indeed due to pheromone-stimulated cell wall breakdown, mutations known to impair cell wall fusion should reduce the frequency of pheromone-induced cell lysis events in the flow chamber. Indeed, deleting *FUS1* and *FUS2* alone and in combination caused more than a 14-fold reduction in cell lysis in the flow chamber when cells were exposed to 50nM α -factor for 5 hours in rich media ($p < 0.002$) (Figure 3.10). This gives further evidence that the cell lysis events in the flow chamber are due to pheromone stimulation as opposed to simply a result of physically constraining growing cells. Since *FUS1* and *FUS2* have also been shown to be involved in cell wall fusion, their role in pheromone-induced cell death supports our hypothesis that the cell lysis observed in the flow chamber is due to breakdown of the cell wall.

DISCUSSION

The mating of budding yeast is risky and elaborately choreographed. When two haploid yeast cells mate, they signal through reciprocal pheromones and receptors, stimulate each other to signal ever more strongly, arrest their cell cycles, use pheromone gradients to direct their

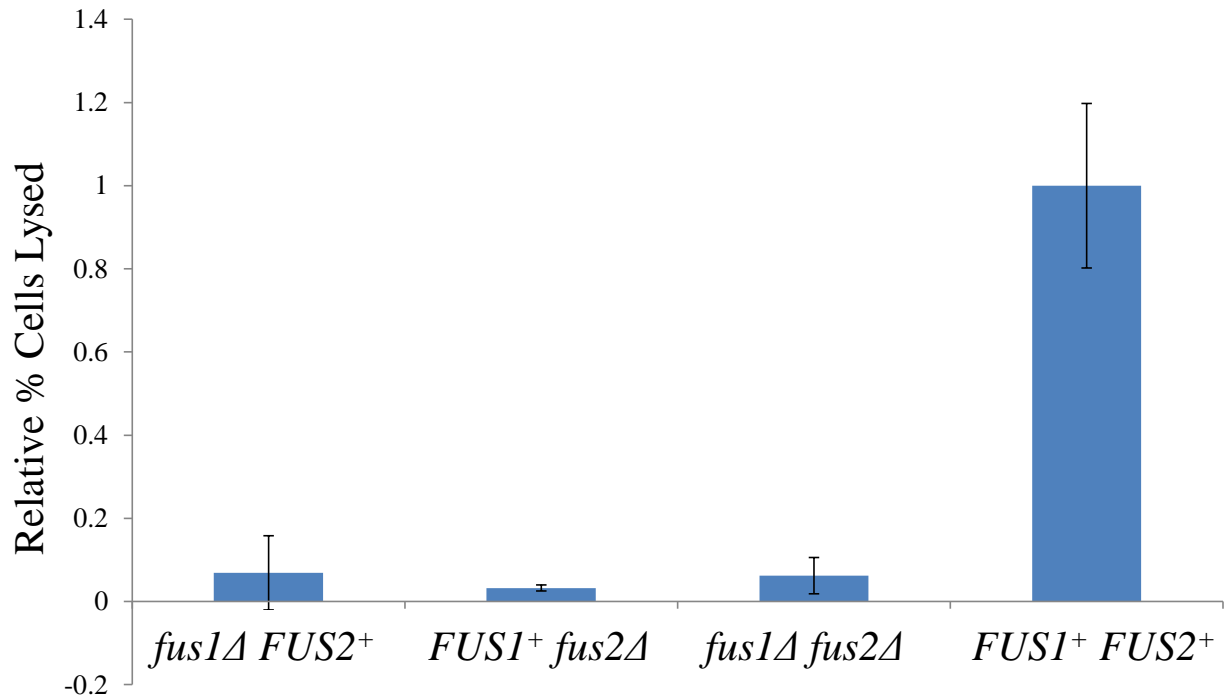


Figure 3.10. The deletion of *FUS1* and *FUS2* mitigates pheromone-induced cell lysis.

Percentage of *MATa bar1Δ* cells deleted for different combinations of *FUS1* and *FUS2* lysed relative to *MATa bar1Δ* cells lysed. Cells were grown in a flow chamber for 5 hours perfused with rich media with 50nM α -factor and imaged using 20x magnification.

polarization towards each other, and eventually fuse their cell walls, cell membranes, and nuclei to form a single diploid cell^{9, 19, 24, 48}. Although many aspects of yeast mating have been well studied, the mechanism by which cells fuse their cell walls is still unclear. Cell wall fusion is a particularly dangerous step in yeast mating. The plasma membranes of the two partner cells cannot touch each other and fuse until the cell walls that lie between them have been dissolved⁹. Because the osmolarity inside a cell is so much higher than outside, the elasticity of the cell wall opposes the osmotic pressure difference between the cytoplasm and the environment, thus keeping water from rushing into the cell and causing it to lyse. A cell that dissolves any part of its cell wall that does not touch a closely apposed mating partner will die^{29, 30}.

Pheromone-induced cell death has been previously attributed to inappropriate cell wall dissolution, but a hypothesis to explain why cells were dissolving their cell walls was not given⁵⁴. Many hypotheses can be generated to explain how cell wall dissolution is regulated in time and space to promote mating and prevent accidental deaths. Most of them posit additional signaling systems in addition to the known mechanisms of pheromone signaling, but these hypothetical, additional signaling molecules have yet to be uncovered despite a variety of searches^{9, 37, 69, 70}. We therefore sought a hypothesis that required no new components but appealed to the physical differences between mating cell pairs and isolated, pheromone-stimulated cells. Our model proposes that growing cells secrete hydrolytic, cell wall-remodeling enzymes that diffuse through the cell wall, breaking bonds within it. These remodeling enzymes weaken the cell wall enough to allow the cell wall expansion required for cell growth but are not present in the cell wall long enough to breach it (Figure 3.2A). Pheromone stimulation induces increased production of cell wall remodeling enzymes and directs their secretion towards the shmoo tip (Figure 3.1B). When two cells are attached via mating agglutinins, it takes longer for the cell

wall remodeling enzymes to diffuse through the two cell walls, allowing the concentration of cell wall remodeling enzymes between the two cells to rise and reducing the thickness of the cell wall. As the wall becomes thinner, the concentration of the enzymes rises further until eventually the wall that separates the shmoo tips has been completely dissolved, exposing the two plasma membranes to each other and allowing their fusion to create a single, diploid cell (Figure 3.2B).

Our data provides strong support for the hypothesis that increased secretion of remodeling enzymes and longer distances for them to diffuse causes cell wall dissolution. By testing cells that are tightly apposed to a non-permeable surface rather than another cell, the only signal these cells can receive is a consistently high concentration of pheromone. The cell lysis events we observe are not due merely to the physical constraints of a flow chamber, since they also occur when the cells are chemically attached to a glass coverslip with essentially infinite space to expand and do not occur when cells are arrested in mitosis by treating them with benomyl, a condition that does not lead to polarized secretion⁶¹. By manipulating the presence of an osmoprotectant in the media, we show that the frequency of lysis events can be increased by increasing the difference between the osmolarity of the cell and its environment and decreased by decreasing the difference between the osmolarity of the cell and its environment, implying that these lysis events are due to a breach in the cell wall. The lysis is specific to pheromone stimulation, as shown by its mitigation when *FUS1* or *FUS2* are deleted, and the cell wall dissolution is at least partially accomplished by the putative glucanases, Scw4 and Scw11, known to be involved in mating.

Taken together with previously published studies, our data supports a model that involves pheromone-induced, polarized secretion of cell wall remodeling enzymes. When cells are pheromone stimulated, a MAP kinase cascade activates transcription of pheromone-induced

genes¹⁹. Along with many others, these genes include the expression of mating agglutinins and cell wall remodeling enzymes, which are packaged into vesicles for secretion into the extracellular environment^{25-27, 31, 32}. Fus2 and Rvs161, a protein that binds to curved membranes^{35, 36} and is involved in cell fusion³⁷, bind to these vesicles and travel along actin cables to the site of polarization in a Myo2-dependent fashion⁷¹, where they are anchored to the plasma membrane by Fus1³⁴, which interacts with the polarisome⁴². Fus2 and Rvs161 in conjunction with Cdc42 may then function to facilitate fusion of these vesicles with the plasma membrane⁷².

When cells are weakly stimulated, they form broad shmoos (Figure 3.6B). Although these cells are polarized, the zone of polarization is relatively large, and presumably, the vesicles containing cell wall remodeling enzymes are released into a relatively large area. The enzymes cleave carbohydrate bonds as they diffuse through the cell wall matrix, weakening the cell wall and allowing for further expansion in the direction of highest pheromone concentration⁴⁶. As a shmoo gets closer to a cell of the opposite mating type, the pheromone concentration increases and the shmoo tip becomes more tightly polarized^{50, 51}. This tighter polarization focuses the secretion of cell wall remodeling enzymes into a smaller fraction of the cell surface, increasing the concentration of cell wall remodeling enzymes in this zone. Although the concentration of cell wall remodeling enzymes in this zone has increased, it is not typically high enough to cause dissolution of the cell wall unless the shmoo tip is pressed against an impermeable barrier, thus slowing the diffusion of the cell wall remodeling enzymes through the cell wall and into the extracellular environment (Figure 3.2A). However, when the two polarized cells attach at their shmoo tips via mating agglutinins, the presence of a second cell membrane traps the remodeling enzymes in the cell wall by requiring them to move laterally along the cell surface to exit the cell wall instead of perpendicularly to the tangent of the curve of the cell, which increases their

concentration and allows for cell wall dissolution (Figure 3.2B). As the cell wall dissolves, the two plasma membranes come into contact with one another, allowing membrane fusion to begin and pushing the Fus2-bound vesicles outward, which allows for the rest of the intervening cell wall to be dissolved and eventually full fusion of the newly formed zygote.

Understanding more about the cell fusion of budding yeast is an important step in understanding cell fusion in more complex organisms. Although animal cells do not have a cell wall, the extracellular matrix surrounding these cells must be dissolved prior to cell fusion. It is possible that the evidence provided here will help to shed light on these other forms of cell fusion.

MATERIALS and METHODS

Yeast strains and culturing

Strains used in this study are listed in Table 3.1. All strains were derived from the W303 wild-type background (*ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1*) using standard genetic techniques. All media was prepared as described in Sherman *et al.*, 1974 and contained 2% wt/vol of glucose⁷³. Cells were either grown in Synthetic Complete media (2% glucose) (SC) or Yeast Extract Peptone Dextrose (2% glucose) (YPD) at 30°C in culture tubes on roller drums or at room temperature (25°C) for timelapse microscopy. Bovine serum albumin (BSA) was used to reduce the non-specific absorption of α -factor to glass and plastic surfaces; it was made into 10% wt/vol stocks in deionized water and then diluted into media to 0.1% wt/vol. Synthetic α -factor (Biosynthesis, Lewisville, TX) was suspended in dimethyl sulfoxide (DMSO) and then diluted into YPD + 0.1% BSA or SC + 0.1% BSA at the appropriate concentration. When appropriate, 1M sorbitol was added to YPD by dissolving sorbitol powder into YPD. YPD containing 1-(butylcarbomoyl)-2-benzimidazolecarbamate (benomyl) was prepared by heating

YPD to 65°C and adding 34mM benomyl in DMSO dropwise to a final concentration of 0.1mM. Peptone, yeast extract, and yeast nitrogen base were obtained from BD (VWR). Bacto-agar was obtained from US Biological (Swampscott, MA). Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich.

Microscopy

Microscopy was done at room temperature using a Nikon Ti-E inverted microscope with a 20x Plan Apo VC 0.75NA air lens, and images were acquired with a Photometrics CoolSNAP HQ camera (Roper Scientific, AZ). Timelapse photography was done using Metamorph 7.7 (Molecular Devices, CA); pictures were acquired every 10 minutes using 10ms exposure for differential interference contrast images and 6ms exposure for brightfield images.

Bulk culture lysis assay

Cells were grown to log phase ($\sim 5 \times 10^6$ cells/mL) at 30°C in YPD and counted using a Z2 Coulter counter (Beckman-Coulter, CA). Cells were washed in YPD + 0.1% BSA and resuspended at 10^6 cells/mL into plastic 14 mL culture tubes (BD Falcon) in YPD + 0.1% BSA with the appropriate concentration of α -factor. These cultures were then incubated on a roller drum at 30°C for 5 hours. Cells were then put directly onto glass slides (Corning) with uncoated coverslips (VWR) and imaged using differential interference contrast. Prior to the experiment, the plastic culture tubes were coated in BSA by incubating overnight at 4°C with phosphate buffered saline (PBS) with 2% wt/vol BSA. The PBS + 2% BSA was poured out immediately prior to the addition of the cell cultures. To determine the percentage of cells that lysed, more than 50 cells were counted from each trial. Statistical significance was determined using Student's *t*-Test.

Concanavalin-A coated coverslip lysis assay

Coverslips (VWR) were coated in concanavalin A (MP Biomedicals, OH) in a protocol modified from Joglekar *et al.*, 2008⁷⁴. Briefly, coverslips were soaked in 1M NaOH for 1 hour at room temperature (25°C), rinsed 5 times with de-ionized, filtered water, and then incubated at room temperature for 1 hour in a solution of 10nM Na₂HPO₄ pH 6.0 (Fisher Biotech) + 1M CaCl₂ + 0.5mg/mL concanavalin A. Coverslips were then rinsed 5 times with de-ionized, filtered water and air-dried over a 100°C heat block. To make a chamber, strips of parafilm (American National Can) were melted at 100°C on glass slides (Corning) and concanavalin A-coated coverslips were placed on top of the strips. The parafilm was allowed to cool to room temperature, creating channels with a glass slide ceiling, concanavalin A-coated coverslip floor, and parafilm walls.

Cells were grown to log phase ($\sim 5 \times 10^6$ cells/mL) at 30°C in YPD and then washed in SC + 0.1% BSA. The appropriate amount of α -factor was added to the cells and the cells were immediately injected into the chamber using capillary action. The cells were allowed to adhere to the concanavalin A-coated coverslip for 10 minutes and then 200 μ L of SC + 0.1% BSA with the appropriate concentration of α -factor was flowed through chamber using capillary action to wash off excess cells. The chamber was then sealed with candle wax and imaged at 20x magnification every 10 minutes for 5 hours from the point of α -factor addition to the cells using differential interference contrast with a 10ms exposure. To determine the percentage of cells that lysed, more than 400 cells were counted from each trial. Statistical significance was determined using Student's *t*-Test.

Flow chamber lysis assay

Cells were grown to log phase ($\sim 5 \times 10^6$ cells/mL) at 30°C in YPD and then washed in YPD + 0.1% BSA. For experiments involving α -factor, the microfluidics chambers (CellAsic,

Hayward, CA)⁷⁵ were pretreated by perfusing PBS + 2% BSA through the chamber at 34kPa for 10 minutes and then YPD + 0.1% BSA through the chamber for 10 minutes. After cells were loaded, YPD + 0.1% BSA with the appropriate concentration of α -factor was perfused through the chamber at 14kPa and pictures were taken at 20x magnification every 10 minutes for 5 hours using differential interference contrast with a 10ms exposure.

For experiments involving benomyl, the microfluidics chambers were pretreated by perfusing YPD through the chamber at 34kPa for 10 minutes. After the cells were loaded, YPD + 0.1mM benomyl was perfused through the chamber at 14kPa and pictures were taken at 20x magnification every 10 minutes for 5 hours using differential interference contrast with a 10ms exposure. The microfluidics chambers were pretreated the same way in experiments with YPD, but after the cells were loaded, YPD was perfused through the chamber at 14kPa and pictures were taken at 20x magnification every 5 minutes for 5 hours using brightfield with 6ms exposure. To determine the percentage of cells that lysed, more than 250 cells were counted from each trial. Statistical significance was determined using Student's *t*-Test.

Sorbitol wash-out assay

Cells were grown to log phase ($\sim 5 \times 10^6$ cells/mL) at 30°C in YPD and then washed in YPD + 0.1% BSA. The microfluidics chambers (CellAsic)⁷⁵ were pretreated by perfusing PBS + 2% BSA through the chamber at 34kPa for 10 minutes and then YPD + 1M sorbitol + 0.1% BSA through the chamber for 10 minutes. After cells were loaded, YPD + 1M sorbitol + 0.1% BSA + 50nM α -factor was perfused through the chamber at 14kPa for 5 hours. After 5 hours, the media containing 1M sorbitol was washed out and YPD + 0.1% BSA + 50nM α -factor was perfused through the chamber at 14kPa for 2 hours. Pictures were taken at 20x magnification every 10 minutes for 7 hours using differential interference contrast with a 10ms exposure. To determine

the percentage of cells that lysed, more than 500 cells were counted from each trial. Statistical significance was determined using Student's *t*-Test.

Sorbitol wash-in assay

Cells were grown to log phase ($\sim 5 \times 10^6$ cells/mL) at 30°C in YPD and then washed in YPD + 0.1% BSA. The microfluidics chambers (CellAsic)⁷⁵ were pretreated by perfusing PBS + 2% BSA through the chamber at 34kPa for 10 minutes and then YPD + 0.1% BSA through the chamber for 10 minutes. After cells were loaded, YPD + 0.1% BSA + 50nM α -factor was perfused through the chamber at 14kPa for 80 minutes. After 80 minutes, the YPD + 0.1% BSA + 50nM α -factor was washed out and YPD + 1M Sorbitol + 0.1% BSA + 50nM α -factor was perfused through the chamber at 14kPa for 60 minutes. Pictures were taken at 20x magnification every 10 minutes for 140 minutes using differential interference contrast with a 10ms exposure. To determine the percentage of cells that lysed, more than 600 cells were counted from each trial. Statistical significance was determined using Student's *t*-Test.

Table 3.1. Strains used in Chapter 3.

Strain Name	Genotype (all cells are in the W303 background)
LBHY52	<i>MATa bar1Δ::KanMX6 P_{ACT1}-yCerulean-HIS3MX6 @ P_{ACT1}</i>
LBHY77	<i>MATa bar1Δ::KanMX6 fus1Δ::NatMX4 P_{ACT1}-yCerulean-HISMX3 @ P_{ACT1}</i>
LBHY80	<i>MATa bar1Δ::KanMX6 fus2Δ::HphMX4 P_{ACT1}-yCerulean-HISMX3 @ P_{ACT1}</i>
LBHY84	<i>MATa bar1Δ::KanMX6 fus1Δ::NatMX4 fus2Δ::HphMX4 P_{ACT1}-yCerulean-HISMX3 @ P_{ACT1}</i>
LBHY120	<i>MATa bar1Δ::ADE2 HIS3:SPA2-YFP scw4Δ::KanMX6 ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1</i>
LBHY121	<i>MATa bar1Δ::ADE2 HIS3:SPA2-YFP scw10Δ::NatMX4 ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1</i>
LBHY136	<i>MATa bar1Δ::ADE2 HIS3:SPA2-YFP scw11Δ::HphMX4 ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1</i>
LBHY138	<i>MATa bar1Δ::ADE2 HIS3:SPA2-YFP scw4Δ::KanMX6 scw10Δ::NatMX4 ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1</i>
LBHY147	<i>MATa bar1Δ::ADE2 HIS3:SPA2-YFP scw4Δ::KanMX6 scw10Δ::NatMX4 scw11Δ::HphMX4 ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1</i>
LBHY153	<i>MATa bar1Δ::ADE2 HIS3:SPA2-YFP scw4Δ::KanMX6 scw11Δ::HphMX4 ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1</i>
LBHY154	<i>MATa bar1Δ::ADE2 HIS3:SPA2-YFP scw10Δ::NatMX4 scw11Δ::HphMX4 ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1</i>
MP 384	<i>MATa bar1Δ::ADE2 HIS3:SPA2-YFP ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1</i>
SLY412	<i>MATa ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1 (W303 wildtype)</i>

References

1. Chen, E. H., Grote, E., Mohler, W. & Vignery, A. Cell-cell fusion. *FEBS Lett.* **581**, 2181-2193 (2007).
2. Ishii, M. & Saeki, Y. Osteoclast cell fusion: mechanisms and molecules. *Mod. Rheumatol.* **18**, 220-227 (2008).
3. Primakoff, P. & Myles, D. G. Penetration, adhesion, and fusion in mammalian sperm-egg interaction. *Science* **296**, 2183-2185 (2002).
4. Chen, E. H. & Olson, E. N. Unveiling the mechanisms of cell-cell fusion. *Science* **308**, 369-373 (2005).
5. Fleissner, A., Simonin, A. R. & Glass, N. L. Cell fusion in the filamentous fungus, *Neurospora crassa*. *Methods Mol. Biol.* **475**, 21-38 (2008).
6. Alper, S. & Podbilewicz, B. Cell fusion in *Caenorhabditis elegans*. *Methods Mol. Biol.* **475**, 53-74 (2008).
7. Wilson, N. F. Gametic cell adhesion and fusion in the unicellular alga *Chlamydomonas*. *Methods Mol. Biol.* **475**, 39-51 (2008).
8. Abmayr, S. M., Zhuang, S. & Geisbrecht, E. R. Myoblast fusion in *Drosophila*. *Methods Mol. Biol.* **475**, 75-97 (2008).
9. Ydenberg, C. A. & Rose, M. D. Yeast mating: a model system for studying cell and nuclear fusion. *Methods Mol. Biol.* **475**, 3-20 (2008).
10. Herskowitz, I. Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiol. Rev.* **52**, 536-553 (1988).
11. Dohlman, H. G. & Thorner, J. W. Regulation of G protein-initiated signal transduction in yeast: paradigms and principles. *Annu. Rev. Biochem.* **70**, 703-754 (2001).
12. Hagen, D. C., McCaffrey, G. & Sprague, G. F., Jr. Evidence the yeast STE3 gene encodes a receptor for the peptide pheromone a factor: gene sequence and implications for the structure of the presumed receptor. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 1418-1422 (1986).
13. Kurjan, J. & Herskowitz, I. Structure of a yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor. *Cell* **30**, 933-943 (1982).
14. Singh, A. *et al.* *Saccharomyces cerevisiae* contains two discrete genes coding for the alpha-factor pheromone. *Nucleic Acids Res.* **11**, 4049-4063 (1983).

15. Blumer, K. J., Reneke, J. E. & Thorner, J. The STE2 gene product is the ligand-binding component of the alpha-factor receptor of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **263**, 10836-10842 (1988).
16. Betz, R. & Duntze, W. Purification and partial characterization of a factor, a mating hormone produced by mating-type-a cells from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **95**, 469-475 (1979).
17. Hartig, A., Holly, J., Saari, G. & MacKay, V. L. Multiple regulation of STE2, a mating-type-specific gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **6**, 2106-2114 (1986).
18. Fields, S., Chaleff, D. T. & Sprague, G. F., Jr. Yeast STE7, STE11, and STE12 genes are required for expression of cell-type-specific genes. *Mol. Cell. Biol.* **8**, 551-556 (1988).
19. Bardwell, L. A walk-through of the yeast mating pheromone response pathway. *Peptides* **25**, 1465-1476 (2004).
20. Zeitlinger, J. *et al.* Program-specific distribution of a transcription factor dependent on partner transcription factor and MAPK signaling. *Cell* **113**, 395-404 (2003).
21. Johnson, G. L. & Lapadat, R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298**, 1911-1912 (2002).
22. Casamayor, A. & Snyder, M. Bud-site selection and cell polarity in budding yeast. *Curr. Opin. Microbiol.* **5**, 179-186 (2002).
23. Butty, A. C., Pryciak, P. M., Huang, L. S., Herskowitz, I. & Peter, M. The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. *Science* **282**, 1511-1516 (1998).
24. Chang, F. & Herskowitz, I. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. *Cell* **63**, 999-1011 (1990).
25. Cappellaro, C., Baldermann, C., Rachel, R. & Tanner, W. Mating type-specific cell-cell recognition of *Saccharomyces cerevisiae*: cell wall attachment and active sites of a- and alpha-agglutinin. *EMBO J.* **13**, 4737-4744 (1994).
26. Roy, A., Lu, C., Marykwas, D., Lipke, P. & Kurjan, J. The AGA1 product is involved in cell surface attachment of the *Saccharomyces cerevisiae* cell adhesion glycoprotein a-agglutinin. *Mol. Cell. Biol.* **11**, 4196-4206 (1991).
27. Zhao, H., Shen, Z., Kahn, P. C. & Lipke, P. N. Interaction of α -agglutinin and a-agglutinin, *Saccharomyces cerevisiae* sexual cell adhesion molecules. *J. Bacteriol.* **183**, 2874-2880 (2001).

28. Dupres, V., Dufrêne, Y. F. & Heinisch, J. J. Measuring cell wall thickness in living yeast cells using single molecular rulers. *ACS nano* **4**, 5498-5504 (2010).
29. Philips, J. & Herskowitz, I. Osmotic balance regulates cell fusion during mating in *Saccharomyces cerevisiae*. *J. Cell Biol.* **138**, 961-974 (1997).
30. de Nobel, H. *et al.* Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in FKS2-lacZ expression, glucanase resistance and thermotolerance. *Microbiology* **146** (Pt 9), 2121-2132 (2000).
31. Brizzio, V., Gammie, A. E., Nijbroek, G., Michaelis, S. & Rose, M. D. Cell fusion during yeast mating requires high levels of a-factor mating pheromone. *J. Cell Biol.* **135**, 1727-1739 (1996).
32. Cappellaro, C., Mersa, V. & Tanner, W. New potential cell wall glucanases of *Saccharomyces cerevisiae* and their involvement in mating. *J. Bacteriol.* **180**, 5030-5037 (1998).
33. Dolan, J. W., Kirkman, C. & Fields, S. The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. *Proceedings of the National Academy of Sciences* **86**, 5703-5707 (1989).
34. Paterson, J. M., Ydenberg, C. A. & Rose, M. D. Dynamic localization of yeast Fus2p to an expanding ring at the cell fusion junction during mating. *J. Cell Biol.* **181**, 697-709 (2008).
35. Friesen, H. *et al.* Characterization of the yeast amphiphysins Rvs161p and Rvs167p reveals roles for the Rvs heterodimer in vivo. *Mol. Biol. Cell* **17**, 1306-1321 (2006).
36. Peter, B. J. *et al.* BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. *Science* **303**, 495-499 (2004).
37. Kurihara, L. J., Beh, C. T., Latterich, M., Schekman, R. & Rose, M. D. Nuclear congression and membrane fusion: two distinct events in the yeast karyogamy pathway. *J. Cell Biol.* **126**, 911 (1994).
38. Bon, E. *et al.* A network of proteins around Rvs167p and Rvs161p, two proteins related to the yeast actin cytoskeleton. *Yeast* **16**, 1229-1241 (2000).
39. Brizzio, V., Gammie, A. E. & Rose, M. D. Rvs161p interacts with Fus2p to promote cell fusion in *Saccharomyces cerevisiae*. *J. Cell Biol.* **141**, 567-584 (1998).
40. Trueheart, J., Boeke, J. D. & Fink, G. R. Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. *Mol. Cell. Biol.* **7**, 2316-2328 (1987).
41. Trueheart, J. & Fink, G. R. The yeast cell fusion protein FUS1 is O-glycosylated and spans the plasma membrane. *Proceedings of the National Academy of Sciences* **86**, 9916-9920 (1989).

42. Nelson, B. *et al.* Fus1p interacts with components of the Hog1p mitogen-activated protein kinase and Cdc42p morphogenesis signaling pathways to control cell fusion during yeast mating. *Genetics* **166**, 67-77 (2004).
43. Sheu, Y., Barral, Y. & Snyder, M. Polarized growth controls cell shape and bipolar bud site selection in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **20**, 5235-5247 (2000).
44. Klis, F. M. Review: cell wall assembly in yeast. *Yeast* **10**, 851-869 (1994).
45. Cid, V. J. *et al.* Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. *Microbiol. Rev.* **59**, 345-386 (1995).
46. Lesage, G. & Bussey, H. Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **70**, 317-343 (2006).
47. Lipke, P. N. & Ovalle, R. Cell wall architecture in yeast: new structure and new challenges. *J. Bacteriol.* **180**, 3735-3740 (1998).
48. Barkai, N., Rose, M. D. & Wingreen, N. S. Protease helps yeast find mating partners. *Nature* **396**, 422-423 (1998).
49. Jackson, C. L. & Hartwell, L. H. Courtship in *S. cerevisiae*: both cell types choose mating partners by responding to the strongest pheromone signal. *Cell* **63**, 1039-1051 (1990).
50. Bagnat, M. & Simons, K. Cell surface polarization during yeast mating. *Proceedings of the National Academy of Sciences* **99**, 14183-14188 (2002).
51. Ayscough, K. R. & Drubin, D. G. A role for the yeast actin cytoskeleton in pheromone receptor clustering and signalling. *Current biology* **8**, 927-931 (1998).
52. Segall, J. E. Polarization of yeast cells in spatial gradients of alpha mating factor. *Proceedings of the National Academy of Sciences* **90**, 8332-8336 (1993).
53. Severin, F. & Hyman, A. Pheromone induces programmed cell death in *S. cerevisiae*. *Current biology: CB* **12**, R233 (2002).
54. Zhang, N. *et al.* Multiple signaling pathways regulate yeast cell death during the response to mating pheromones. *Mol. Biol. Cell* **17**, 3409-3422 (2006).
55. Sprague Jr, G. F. & Herskowitz, I. Control of yeast cell type by the mating type locus: I. Identification and control of expression of the a-specific gene BAR1. *J. Mol. Biol.* **153**, 305-321 (1981).
56. Chan, R. K. & Otte, C. A. Physiological characterization of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by a factor and alpha factor pheromones. *Mol. Cell. Biol.* **2**, 21 (1982).

57. Terrance, K. & Lipke, P. N. Sexual agglutination in *Saccharomyces cerevisiae*. *J. Bacteriol.* **148**, 889-896 (1981).
58. Voth, W. P., Olsen, A. E., Sbia, M., Freedman, K. H. & Stillman, D. J. ACE2, CBK1, and BUD4 in budding and cell separation. *Eukaryotic cell* **4**, 1018-1028 (2005).
59. Biely, P., Kratky, Z. & Bauer, S. Interaction of concanavalin A with external mannan-proteins of *Saccharomyces cerevisiae*. Glycoprotein nature of beta-glucanases. *Eur. J. Biochem.* **70**, 75-81 (1976).
60. Johnston, G., Pringle, J. & Hartwell, L. Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.* **105**, 79-98 (1977).
61. Li, R. & Murray, A. W. Feedback control of mitosis in budding yeast. *Cell* **66**, 519-531 (1991).
62. Hutchison, H. T. & Hartwell, L. H. Macromolecule synthesis in yeast spheroplasts. *J. Bacteriol.* **94**, 1697-1705 (1967).
63. Brown, A. Compatible solutes and extreme water stress in eukaryotic micro-organisms. *Adv. Microb. Physiol.* **17**, 181-242 (1978).
64. Blomberg, A. & Adler, L. Physiology of osmotolerance in fungi 1. *Adv. Microb. Physiol.* **33**, 145 (1992).
65. Hirayama, T., Maeda, T., Saito, H. & Shinozaki, K. Cloning and characterization of seven cDNAs for hyperosmolarity-responsive (HOR) genes of *Saccharomyces cerevisiae*. *Molecular and General Genetics MGG* **249**, 127-138 (1995).
66. O'Rourke, S. M., Herskowitz, I. & O'Shea, E. K. Yeast go the whole HOG for the hyperosmotic response. *Trends Genet.* **18**, 405-412 (2002).
67. Colman-Lerner, A., Chin, T. E. & Brent, R. Yeast Cbk1 and Mob2 activate daughter-specific genetic programs to induce asymmetric cell fates. *Cell* **107**, 739-750 (2001).
68. Sestak, S., Hagen, I., Tanner, W. & Strahl, S. Scw10p, a cell-wall glucanase/transglucosidase important for cell-wall stability in *Saccharomyces cerevisiae*. *Microbiology* **150**, 3197-3208 (2004).
69. Gammie, A. E., Brizzio, V. & Rose, M. D. Distinct morphological phenotypes of cell fusion mutants. *Mol. Biol. Cell* **9**, 1395-1410 (1998).
70. Marsh, L. & Rose, M. D. 10 The Pathway of Cell and Nuclear Fusion during Mating in *S. cerevisiae*. *Cold Spring Harbor Monograph Archive* **21**, 827-888 (1997).

71. Sheltzer, J. M. & Rose, M. D. The class V myosin Myo2p is required for Fus2p transport and actin polarization during the yeast mating response. *Mol. Biol. Cell* **20**, 2909-2919 (2009).
72. Ydenberg, C. A., Stein, R. A. & Rose, M. D. Cdc42p and Fus2p act together late in yeast cell fusion. *Mol. Biol. Cell* **23**, 1208-1218 (2012).
73. Sherman, F., Fink, G. & Lawrence, C. in *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory Press, New York, 1974).
74. Joglekar, A. P., Salmon, E. & Bloom, K. S. Counting kinetochore protein numbers in budding yeast using genetically encoded fluorescent proteins. *Methods Cell Biol.* **85**, 127-151 (2008).
75. Lee, P. J., Helman, N. C., Lim, W. A. & Hung, P. J. A microfluidic system for dynamic yeast cell imaging. *BioTechniques* **44**, 91-95 (2008).

CHAPTER 4

Conclusions, discussion, and future directions

ABSTRACT

Mating in the budding yeast is a complex, highly regulated process that mirrors many of the processes in the cell biology of higher eukaryotes. The studies presented in this thesis serve to elucidate several aspects of intercellular communication and fusion. However, they also bring to light additional questions and possible future directions for research. These include further characterization of the biological and biochemical functions of the **a**-factor barrier protein, Afb1, and further investigation of the differences in the regulation of gene expression in *MATa* and *MATα* cells as well as more studies on how robust the process of intercellular communication and fusion is. Additionally, while we have provided support for the hypothesis that cell wall dissolution occurs through contact-limited diffusion of cell wall remodeling enzymes, we have not negated the possibility that additional signaling pathways are involved, and additional experiments could be done to provide further support for our hypothesis. Through the future directions described here and elsewhere, we can hope that eventually the complex process of the mating of the budding yeast will be completely elucidated.

INTRODUCTION

The study of mating in the budding yeast, *Saccharomyces cerevisiae*, is an excellent model system for many of the processes we see in higher eukaryotes. Signaling through G-protein coupled receptors and subsequent activation of a mitogen-activated protein (MAP) kinase signaling cascade is relevant to intercellular communication that regulates growth and division¹. Cell polarization in an external gradient of signaling molecules is similar to the maturation of neuron axons in the development and maintenance of the nervous system². The process by which yeast cells arrest the cell cycle in response to pheromone can tell us more about the control of the cell cycle in general.

Budding yeast cells are also capable of differentiating into three different cell types: *MATa*, *MAT α* , and *MATa/ α* ^{ref. 3}. This process is one of the simplest and most easily controllable examples of cellular differentiation, making it an excellent model system. Studying the differentially expressed genes in these different cell types is a useful way to determine which genes are necessary to define a cell type. Since the differentiation between *MATa* and *MAT α* cells mainly concerns differences necessary for mating⁴, investigating how the careful regulation of these genes controls mating efficiency can also inform us about the robustness of intercellular communication in this system. In Chapter 2, I discussed the characterization of transvestite yeast: yeast cells whose mating type-specific transcription is under the control of one set of transcription factors at the *MAT* locus but express the mating type-specific genes of the other mating type. The transvestite yeast enabled us to identify the long sought-after **a**-factor barrier protein, *AFB1*. We also observed that pheromone-stimulated *MAT α* cells expressing the α -factor receptor, Ste2, shmoo but arrest in G1 only transiently. It is possible that this indicates a novel mechanism through which *MAT α* cells attenuate the cell cycle in response to pheromone-

stimulation either in general or specifically through Ste2. With respect to *MATa* cells, we gave further evidence to support the hypothesis that all of the *MATa*-specific genes have been identified since it was possible to achieve mating efficiencies at near wild-type levels in the *a*-playing- α *P_{TDH3}-MF α 1* cells. Additionally, the transvestite yeast give us a tantalizing view into the robustness of the intercellular communication in budding yeast mating.

Once two haploid cells have successfully signaled and polarized towards each other, they must go through cell fusion to form a single diploid zygote. This process has relevance to the fertilization of an egg by sperm as well as formation of muscle cells, osteoclasts, giant cells, and placental cells⁵⁻⁸. Since the budding yeast nucleus is incased in a series of 3 concentric envelopes, generation of a diploid cell requires a series of three types of fusion: cell wall fusion, plasma membrane fusion, and nuclear fusion⁹. Each of these is a tightly regulated process ensuring that the various compartments of the cell stay isolated from each other and from the extracellular environment. In Chapter 3 we provided support for the hypothesis that yeast cells limit cell wall dissolution to the point of contact with a fusion partner via contact-limited diffusion of cell wall remodeling enzymes. We hypothesize that pheromone-stimulated cells secrete cell wall remodeling enzymes to remodel their cell walls and enable polarized growth of a shmoo. When cells are unattached, these cell wall remodeling enzymes can diffuse into the extracellular environment, weakening but not breaching the cell wall, and thus allowing its continuous expansion as two partner cells grow towards each other. However, when two shmoo tips are attached to each other in preparation for fusion, the cell wall remodeling enzymes are trapped between the shmoo tips, increasing the concentration of cell wall remodeling enzymes, and leading to cell wall dissolution. Cell death due to cell wall dissolution has previously been seen in populations of pheromone-stimulated cells¹⁰, and we showed that this is likely due to

tight apposition of shmoo tips to non-permeable surfaces such as another cell of the same mating type or a glass coverslip, resulting in an increase in the concentration of cell wall remodeling enzymes at the shmoo tip.

The experiments described in this thesis present several important conclusions about the mating of budding yeast and strive to further elucidate the process by which the two haploid cell types communicate and fuse to form a single diploid. However, the conclusions drawn also bring to light the need for future experiments to provide additional information about the mechanisms involved in budding yeast mating.

DISCUSSION and FUTURE DIRECTIONS

Further characterization of AFB1

In Chapter 2 we identified the novel, *MAT* α -specific **a**-factor barrier protein, *AFB1*. When expressed under the endogenous promoter in α -playing-**a** cells, Afb1 causes decreased **a**-factor secretion. We hypothesize that this protein is secreted due to its ability to also block **a**-factor expressed from *MAT***a** cells in mixtures of *MAT***a** and α -playing-**a** cells, and identified the gene as the uncharacterized open reading frame (ORF) *YLR040C*. Since the activity of Afb1 is active in α -playing-**a** cells and in *MAT***a** cells expressing Afb1 under a constitutive promoter, it is unlikely that Afb1 acts to increase endocytosis of **a**-factor receptors bound to **a**-factor. Although we were able to see biological activity of this protein, the specific biochemical activity is still unknown. In 1991, Marcus *et al.* reported the biochemical activity of a *MAT* α -specific **a**-factor endopeptidase¹¹. However, the gene encoding this endopeptidase has not been identified¹².

It is possible that Afb1 is the endopeptidase seen by Marcus *et al.*¹¹. If so, cells expressing Afb1 should be capable of **a**-factor degradation and cells lacking Afb1 should not. If Afb1 does not show endopeptidase activity, it is also possible that it acts in some other way –

perhaps either by cleaving the farnesyl moiety or by somehow sequestering **a**-factor away from Ste3 receptors.

While we did see an increase in the mating efficiency of α -playing-**a** cells lacking Afb1 compared to α -playing-**a** cells that express Afb1 under the endogenous promoter, we did not detect a difference in the mating efficiency of wild-type *MAT α* cells expressing and lacking Afb1. It seems likely that Afb1 would have an effect on mating efficiency under certain conditions, and it would be interesting to identify these. Since Afb1 is hypothesized to have a glycosylphosphatidylinositol (GPI) anchor¹³ keeping it associated with the plasma membrane of *MAT α* cells, perhaps, like Bar1 trapped in the cell walls of *MAT α* cells, Afb1 increases the ability of *MAT α* cells to choose between two nearby potential partners by inhibiting saturation of the pheromone receptors and increasing the ability of cells to identify the specific location of the pheromone source¹⁴. For example, in an ascus the distance between a given germinating *MAT α* spore and the two germinating *MAT α* spores may be close to equidistant, so identifying the exact location of each cell as opposed to an average of the two point signals, resulting in polarization of the *MAT α* cell between the two *MAT α* cells, would be extremely important. It is also possible that, like Bar1 for *MAT α* cells, Afb1 creates **a**-factor sinks around *MAT α* cells in dense mating mixtures, causing *MAT α* cells to polarize away from each other and increasing the likelihood of *MAT α* cells polarizing towards different potential *MAT α* partners¹⁵. Modeling the effect of Afb1 on mating in the ascus and in dense mating mixtures could shed some light on these possibilities.

Another way to investigate the effects of Afb1 on mating efficiency would be to look into whether *MAT α* cells lacking Afb1 are as capable of distinguishing between potential *MAT α* partners as wild-type *MAT α* cells. Jackson and Hartwell, 1990 observed that *MAT α* cells have a preference for *MAT α* cells that produce more **a**-factor¹⁶. It is possible that while *MAT α* cells

lacking Afb1 do not have a mating defect in standard mating assays, they are deficient in partner discrimination. Further elucidation of the role of Afb1 in mating will serve to paint a more complete picture of intercellular communication in the mating of budding yeast.

How robust is mating?

The experiments described in Chapter 2 mainly focused on the mating of the transvestite yeast to wild-type partners and what that can tell us about the different cell types and mating in yeast. However, the low mating efficiency of the two transvestites to each other indicates that defects in mating are synergistic. This result was supported by the fact that crossing the α -playing-**a** cells with “pseudo”-**a**-playing- α cells (*MAT α mfa1 Δ* cells) resulted in similarly low mating efficiency. Perhaps it is not surprising that affecting the regulation of the approximately 130 genes that are differentially regulated in *MAT α* and *MAT α* cells would cause such a large decrease in mating efficiency, but it raises the question of how robust the process of mating is. It is clear that yeast have evolved an efficient mechanism for mating. It has also been shown that sterile cells can outcompete cells capable of mating when competed for purely vegetative growth¹⁷. It would be interesting to continue to investigate whether yeast have evolved to be the best maters possible or if the regulation of sexual and asexual genes is a tradeoff between mating efficiency and asexual generation time. It has been shown that it is possible to evolve cells with faster asexual growth rates¹⁷, but is it possible to engineer a super-mater?

Investigating strains of yeast that produce differing amounts of pheromone would be a simple way to begin to untangle the tradeoffs inherent in sexual and asexual reproduction. We demonstrate in Chapter 2 that cells are capable of producing more α -factor than is typically produced in wild-type *MAT α* cells. This increase in α -factor production did result in an increase in the mating efficiency of the **a**-playing- α cells. However, the **a**-playing- α cells started with

lower than normal α -factor production, they may have additional complexities that affect their mating efficiency, and they did not improve their mating efficiency above typical wild-type levels. This leaves the question of whether additional α -factor production in wild-type *MAT α* cells would improve mating efficiency in traditional mating assays. *MAT \mathbf{a}* cells are capable of discriminating between *MAT α* cells that produce differing amounts of α -factor and prefer wild-type *MAT α* cells to *MAT α* cells deleted for one of the α -factor genes¹⁸. Will *MAT α* cells with higher than normal α -factor production be able to outcompete wild-type *MAT α* cells for a *MAT \mathbf{a}* partner or will the increased α -factor production of these strains overwhelm the ability of *MAT \mathbf{a}* cells to distinguish between potential partners? If *MAT \mathbf{a}* cells do prefer *MAT α* cells with higher than normal α -factor production, it would also be informative to investigate whether this increase in mating efficiency causes a decrease in asexual fitness. Either way, it will be interesting to investigate the tradeoffs inherent in sexual versus asexual reproduction.

How different is the pheromone response in MAT \mathbf{a} and MAT α cells?

The response of *MAT \mathbf{a}* cells to pheromone has been extremely well characterized through the investigation of the *MAT \mathbf{a}* transcriptome under the influence of differing α -factor concentrations and incubation times¹⁹. Because of the hydrophobicity of \mathbf{a} -factor the intense reciprocal investigation of the pheromone-stimulated *MAT α* transcriptome has not been done. The engineering of α -playing- \mathbf{a} cells makes it possible to perform a close comparison of the response of *MAT \mathbf{a}* and *MAT α* cells to pheromone over time and pheromone concentration. Even under the single pheromone concentration and time point tested in Chapter 2, we saw evidence of differential expression in over 100 genes. It is possible that a more thorough investigation of the pheromone-stimulated α -playing- \mathbf{a} transcriptome will identify additional evidence of differential regulation of the pheromone response between *MAT \mathbf{a}* and *MAT α* cells.

When investigating the phenotypic differences between *MATa* and α -playing-**a** cells, we also saw a difference with respect to pheromone-stimulated G1 arrest. While *MATa bar1Δ* cells shmoo and arrest in G1, α -playing-**a** *bar1Δ* cells shmoo but arrest only transiently. We were unable to identify a single gene responsible for this effect and showed that cells expressing both *MATa* and *MATa* specific genes (aside from *BAR1*, *STE3*, *MFa1*, and *MFa2*) are capable of arresting in response to pheromone. A possible explanation for this is that the lack of G1 arrest in pheromone-stimulated *MATa* cells is actually due to a complex interaction of the many differentially regulated genes in *MATa* and *MATa* cells. These many small changes in gene expression could act together to lead to continued budding during pheromone stimulation either in *MATa* cells in general or only in those *MATa* cells expressing the α -factor receptor, Ste2.

It is also, of course, possible that this effect is due to a gene we did not investigate. In 1989, Steden *et al.* identified a mutation that causes increased pheromone-sensitivity specifically in *MATa* cells exposed to **a**-factor and mapped it to a 125 kilobase region on Chromosome XII²⁰, which contains 72 ORFs, none of which are differentially expressed between pheromone-stimulated *MATa* and α -playing-**a** cells. However, perhaps one of these genes interacts with a differentially regulated gene in a way that allows *MATa* cells to continue budding even while pheromone-stimulated. Whether a specific gene is responsible for the lack of enduring cell cycle arrest in α -playing-**a** cells or whether it is the combination of a complex network of differentially expressed genes, a careful investigation of the differences in the transcriptomes of *MATa* and α -playing-**a** cells throughout a variety of times and pheromone-concentrations could be extremely enlightening.

Artificial cell fusion

In order for two haploid cells to fuse to form a single diploid, they must polarize towards each other, attach to each other at their shmoo tips, and dissolve their cell walls specifically at the point of contact. This is an extremely dangerous task because if the cell wall is dissolved at the wrong time or in the wrong location the cell wall will no longer be able to resist the turgor pressure of the cell leading to lysis^{9, 21}. In Chapter 3, we present data supporting our hypothesis that cell wall dissolution is the result of the limited diffusion of secreted cell wall remodeling enzymes when a shmoo tip is attached to a non-permeable surface such as another cell or a glass coverslip.

It is possible to imagine other hypotheses for cell wall dissolution during mating that include additional signaling or regulation, but these additional signaling molecules have yet to be identified. For instance, another hypothesis that fits our data is that pheromone-stimulated cells are able to sense that they are physically touching a non-permeable surface, causing an increase in the secretion of cell wall remodeling enzymes at the point of contact.

A possible future experiment that would provide additional support for our hypothesis would be to artificially fuse two unstimulated cells. One way to do this would be to artificially polarize isotropically growing cells, perhaps using a protein involved in bud polarity, such as Bud5²². Mating agglutinins from both mating types and cell wall remodeling enzymes thought to be involved with mating could be placed under an inducible promoter, and their transcription could be activated as the cells were stimulated for artificial polarization. Since secretion would be polarized to this site, the agglutinins would preferentially be sent here, thus, causing cells to attach specifically by their sites of artificial polarization. The secretion of the cell wall remodeling enzymes would also be polarized to this location, so it might be possible to see cell wall dissolution by looking for the absence of fluorescently labeled cell wall separating two

fluorescently labeled cytoplasms. The ability to fuse the cell walls of cells in the absence of pheromone would argue strongly that additional signaling beyond the pheromone response pathway is unnecessary for cell wall dissolution.

If it is possible to induce yeast cells to fuse their cell walls in the absence of pheromone, it could also be elucidating to ask whether it is possible to artificially induce plasma membrane fusion. Although additional proteins may still need to be identified, it would be interesting to observe the effect of inducing expression of proteins known to be involved in plasma membrane fusion, such as *Prm1*^{ref. 23}, *Fig1*^{ref. 24}, *Kex2*^{ref. 25}, *Erg2*, *Erg3*, and *Erg6*^{ref. 26}. It has been reported that strong pheromone induction is not necessary for plasma membrane fusion²⁶, indicating that if the proteins necessary for fusion were induced, artificial plasma membrane fusion could potentially be possible. If so, artificial plasma membrane fusion could serve to demonstrate that all of the pheromone-induced proteins necessary for plasma membrane fusion had been identified.

CLOSING REMARKS

Our results serve to fill in some of the gaps in our knowledge of the mating of the budding yeast. We have identified a novel *a*-factor barrier protein, *Afb1*, which may serve to clarify the process by which *MATa* and *MATα* cells successfully communicate in both a dense mating mixture and when cells are relatively isolated. We have also provided further evidence that all of the *MATa*-specific genes have been identified and that the process of intercellular communication requires careful regulation of multiple genes to proceed efficiently.

Additionally, we have put forth a hypothesis for the mechanism by which mating partners safely proceed through cell wall fusion. Learning more about how budding yeast cells are able to communicate and eventually fuse is applicable to similar processes in higher eukaryotes as well

as to the mating and fusion of other fungi. Future studies will serve to further elucidate this process, perhaps eventually culminating in a complete understanding of all of the complex interactions surrounding the mating of the budding yeast.

References

1. Luttrell, L. 'Location, location, location': activation and targeting of MAP kinases by G protein-coupled receptors. *J. Mol. Endocrinol.* **30**, 117-126 (2003).
2. Schwamborn, J. C. & Püschel, A. W. The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. *Nat. Neurosci.* **7**, 923-929 (2004).
3. Haber, J. E. Mating-type gene switching in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **32**, 561-599 (1998).
4. Galgoczy, D. J. *et al.* Genomic dissection of the cell-type-specification circuit in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences* **101**, 18069-18074 (2004).
5. Chen, E. H., Grote, E., Mohler, W. & Vignery, A. Cell-cell fusion. *FEBS Lett.* **581**, 2181-2193 (2007).
6. Ishii, M. & Saeki, Y. Osteoclast cell fusion: mechanisms and molecules. *Mod. Rheumatol.* **18**, 220-227 (2008).
7. Primakoff, P. & Myles, D. G. Penetration, adhesion, and fusion in mammalian sperm-egg interaction. *Science* **296**, 2183-2185 (2002).
8. Chen, E. H. & Olson, E. N. Unveiling the mechanisms of cell-cell fusion. *Science* **308**, 369-373 (2005).
9. Ydenberg, C. A. & Rose, M. D. Yeast mating: a model system for studying cell and nuclear fusion. *Methods Mol. Biol.* **475**, 3-20 (2008).
10. Zhang, N. *et al.* Multiple signaling pathways regulate yeast cell death during the response to mating pheromones. *Mol. Biol. Cell* **17**, 3409-3422 (2006).
11. Marcus, S., Xue, C., Naider, F. & Becker, J. Degradation of a-factor by a *Saccharomyces cerevisiae* alpha-mating-type-specific endopeptidase: evidence for a role in recovery of cells from G1 arrest. *Mol. Cell. Biol.* **11**, 1030-1039 (1991).
12. Michaelis, S. & Barrowman, J. Biogenesis of the *Saccharomyces cerevisiae* Pheromone a-Factor, from Yeast Mating to Human Disease. *Microbiology and Molecular Biology Reviews* **76**, 626-651 (2012).
13. Hamada, K., Terashima, H., Arisawa, M., Yabuki, N. & Kitada, K. Amino acid residues in the ω -minus region participate in cellular localization of yeast glycosylphosphatidylinositol-attached proteins. *J. Bacteriol.* **181**, 3886-3889 (1999).

14. Rappaport, N. & Barkai, N. Disentangling signaling gradients generated by equivalent sources. *J. Biol. Phys.* **38**, 267-278 (2012).
15. Jin, M. *et al.* Yeast dynamically modify their environment to achieve better mating efficiency. *Science signaling* **4**, ra54 (2011).
16. Jackson, C. L. & Hartwell, L. H. Courtship in *Saccharomyces cerevisiae*: an early cell-cell interaction during mating. *Mol. Cell. Biol.* **10**, 2202-2213 (1990).
17. Lang, G. I., Murray, A. W. & Botstein, D. The cost of gene expression underlies a fitness trade-off in yeast. *Proceedings of the National Academy of Sciences* **106**, 5755-5760 (2009).
18. Jackson, C. L. & Hartwell, L. H. Courtship in *S. cerevisiae*: both cell types choose mating partners by responding to the strongest pheromone signal. *Cell* **63**, 1039-1051 (1990).
19. Roberts, C. J. *et al.* Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles. *Science Signaling* **287**, 873 (2000).
20. Steden, M., Betz, R. & Duntze, W. Isolation and characterization of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by the mating hormone a-factor. *Molecular and General Genetics MGG* **219**, 439-444 (1989).
21. Lesage, G. & Bussey, H. Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **70**, 317-343 (2006).
22. Chant, J., Corrado, K., Pringle, J. R. & Herskowitz, I. Yeast *BUD5*, encoding a putative GDP-GTP exchange factor, is necessary for bud site selection and interacts with bud formation gene *BEM1*. *Cell* **65**, 1213-1224 (1991).
23. Heiman, M. G. & Walter, P. Prm1p, a pheromone-regulated multispinning membrane protein, facilitates plasma membrane fusion during yeast mating. *J. Cell Biol.* **151**, 719-730 (2000).
24. Aguilar, P. S., Engel, A. & Walter, P. The plasma membrane proteins Prm1 and Fig1 ascertain fidelity of membrane fusion during yeast mating. *Mol. Biol. Cell* **18**, 547-556 (2007).
25. Heiman, M. G., Engel, A. & Walter, P. The Golgi-resident protease Kex2 acts in conjunction with Prm1 to facilitate cell fusion during yeast mating. *J. Cell Biol.* **176**, 209-222 (2007).
26. Jin, H., McCaffery, J. M. & Grote, E. Ergosterol promotes pheromone signaling and plasma membrane fusion in mating yeast. *J. Cell Biol.* **180**, 813-826 (2008).

APPENDIX

Supplemental movies

Movie A1. *MATa* cells are capable of enduring arrest in response to pheromone stimulation. *MATa bar1Δ* cells were grown in a flow chamber for 8 hours perfused with rich media with 10nM α -factor and imaged with DIC every 10 minutes using 60x magnification. The scale bar indicates 10 μ m.

Movie A2. α -playing-a cells arrest only transiently in response to pheromone stimulation. α -playing-a *bar1Δ* cells were grown in a flow chamber for 8 hours perfused with rich media with 10nM α -factor and imaged with DIC every 10 minutes using 60x magnification. The scale bar indicates 10 μ m.

Movie A3. Pheromone stimulated cells lyse when tightly apposed to a non-permeable surface. *MATa bar1Δ* cells were grown in a flow chamber for 5 hours perfused with rich media with 50nM α -factor and imaged every 10 minutes using 20x magnification. The scale bar indicates 10 μ m.

Movie A4. 1M sorbitol protects cells from pheromone-induced cell lysis. Cells were grown in a flow chamber for 7 hours. For the first 5 hours the chamber was perfused with rich media with 1M sorbitol and 50nM α -factor. At the end of 5 hours, the media in the chamber was replaced with rich media with 50nM α -factor and no sorbitol. Cells were imaged every 10 minutes using 20x magnification. The scale bar indicates 10 μ m.